Activation of Methionine Synthetase by a Reduced Triphosphopyridine Nucleotide-dependent Flavoprotein System*

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SUMMARY

Two flavoproteins, both of which are required for the TPNH- and adenosylmethionine-dependent activation of the B12-containing methionine synthetase, have been purified to homogeneity from *Escherichia coli* K-12. The larger flavoprotein (mol wt 27,000) contains 1 mole of noncovalently bound FAD per mole of protein and has an atypical spectrum ($\lambda_{\text{max}}$ at 400 and 456 nm). The smaller flavoprotein (mol wt 19,400) is acidic, contains 1 mole of noncovalently bound FMN per mole of protein, and has absorbance maxima at 369 and 465 nm. The methionine synthetase, which was also purified to homogeneity from *E. coli* K-12, contains 1 mole of B12 per mole of protein (mol wt 186,000) and has an absorbance maximum at 474 nm. In the presence of TPNH, both flavoproteins, and adenosylmethionine, the synthetase has a specific activity at 37°C of 3.8 μmoles per min per mg of protein with respect to formation of methionine from 5-methyltetrahydrofolate and homocysteine.

The following were obtained from commercial sources: AdoMet-chloride (used for stabilization of the M component during purification), p-l-homocysteine thiolactone, dithioerythritol, FMN, FAD, DPNH, TPNH, protamine sulfate, cytochrome c, myoglobin, peptin, serum albumin, urease, alcohol dehydrogenase, a-globulin, dihydrolipoate dehydrogenase, B-galactosidase, and pyruvate kinase (Sigma); AdoMet-iodide (used for enzyme assays) (Calbiochem); a-chymotrypsinogen, trypsin, ovalbumin, and phosphorylase a ( Worthington); glyceraldehyde 3-phosphate dehydrogenase (Boehringer Mannheim); analytical grade KH2PO4 and K3HPO4 ( Mallinckrodt); cellulose and silica thin layer chromatography plates (MN-polygram CEL 300 and SIL 8) (Brinkmann Instruments, Inc.); blue dextran 2000, Sephadex G-50 (fine), G-75 (fine), and DEAE-Sephadex A-50 (Pharmacia); hydroxyapatite (Bio-Gel HTP), Bio-Gel P-200 (100 to 200 mesh), and Bio-Rad resin AG 1-X8 (200 to 400 mesh) (Bio-Rad Laboratories); DEAE-cellulose (microgranular) (Whatman); DEAE-cellulose, DEAE-Sephadex, and hydroxyapatite were suspended in water, deoxygenated several times, washed with 100 mM KPO4 followed by water, and stored at 4°C. Glass-distilled water was used for this purpose and for reagent solutions. Prior to chromatography, each column was equilibrated with the starting buffer.

* The abbreviations used are: AdoMet, S-adenosylmethionine; KPB, potassium phosphate buffer (pH 7.0); PDP, a component of polyvinyl pyrrolidone; and B12, cob(II)alamin.

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thiolactone by the procedure of Hatch et al. (16). Solutions of homocysteine (0.1 M) and dithioerythritol (0.2 M) were stored at 4°C under argon in Thunberg tubes.

**Methods**

Absorbance spectra were obtained with a Cary recording spectrophotometer, model 14. Electrophoresis of proteins on polyacrylamide gel was carried out according to the methods of Ornstein (17) and Davis (18). Staining was achieved with Amido black, followed by electrophoretic destaining using a Canuco rapid destainer.

Analysis of flavins was performed by (a) thin layer chromatography (19) on Sil E plates with 6% Na2HPO4 as the solvent system or on CEL plates with butanol-water-acetic acid-ethanol (7:1.9:5.5:3) or t-butyl alcohol-water (6:4) as the solvent systems; and (b) paper electrophoresis on Whatman No. 3 MM filter paper with a 0.05 M sodium acetate buffer, pH 5.1, as the solvent system (20). Flavins were located by their fluorescence under ultraviolet light.

Molecular weights of proteins were determined by gel filtration according to Whitaker's method (21) or by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels by means of the procedures of Weber and Osborn (22) and Dunker and Riedeker (23).

In the gel filtration method, Bio-Gel P-200, Sephadex G-75, and Sephadex G-50 were used for the M, R, and F components, respectively. Puruvate kinase, γ-globulin, alcohol dehydrogenase, and dihydrolipoate dehydrogenase served as standard proteins for the Bio-Gel P-200 column, while cytochrome c, myoglobin, a-chymotrypsinogen, trypsin, and pepsin served as standards for both Sephadex columns. For all three columns, the void volume was determined with blue dextran 2000. In the sodium dodecyl sulfate-polyacrylamide gel method, proteins were stained with Coomassie blue. The M component was examined in a 5% polyacrylamide gel prepared according to Dunker and Riedeker (23). For the R and F proteins, 10% polyacrylamide gels with 0.75% of the normal amount (22) of cross-linking were employed.

**Assay Systems**

**M Component**

Methionine formation from 5-methyltetrahydrofolate and homocysteine, catalyzed by the M component, was measured by the following methods which are based upon the procedure of Weisbach et al. (24).

**Assay A: Enzymatic Reducing System**—The assay mixture contained, in 0.25 ml, 0.2 μmole of 5-methyltetrahydrofolate (5-MTHF), 3.0 μmoles of homocysteine, 2.0 μmoles of AdoMet-chloride, 10 μmoles of B12, 0.025 μmole of TPNH (or 2 μmoles of DPNH), 10 μmoles of KPB, and the indicated amounts of R, F, and M components. Reactions were performed in conical tubes. For all columns, the void volume was determined with blue dextran 2000. In the sodium dodecyl sulfate-polyacrylamide gel method, proteins were stained with Coomassie blue. The M component was examined in a 5% polyacrylamide gel prepared according to Dunker and Riedeker (23). For the R and F proteins, 10% polyacrylamide gels with 0.75% of the normal amount (22) of cross-linking were employed.

**Assay B: Chemical Reducing System**—This was similar to the enzymatic system, except that TPNH and the R and F components were replaced by 6 μmoles of dithioerythritol.

**R Component**

The activity of the R component was determined either by its DPNH-cytochrome c reductase activity or its ability in the presence of TPNH (or DPNH) and the F component to support methionine synthesis catalyzed by the M component.

For measurement of the cytochrome c reductase activity, the assay mixture contained, in 0.5 ml, 150 μg of cytochrome c, 0.30 μmole of DPNH, 25 μmoles of KPB, and the indicated amount of R component; the blank was identical except for the omission of enzyme. The absorbance change was monitored at 550 nm with the use of a Gilford multiple sample absorbance recorder at 5°C. Results were expressed in absorbance units in absorbance units (absorption maxima) during the initial, linear portion of the curve (0.5 to 2 min). One unit of activity was defined as the amount of enzyme required to produce a ΔA550 of 1.0 per min. Specific activity was expressed as units per mg of protein.

The activity of the R component in support of methionine synthesis was measured by means of Assay System A with fixed amounts of M and F and the R component as a limiting factor. Under those conditions, one unit of R activity was defined as the amount required for the synthesis of 1 μmole of methionine per 15 min. Specific activity was expressed as units per mg of R protein.

**F Component**

This component was also measured under conditions in which it was the limiting factor in Assay A. The volume of the assay mixture was 0.25 or 0.125 ml, but the concentrations of all reactants were unchanged. Activity and specific activity were defined in a manner similar to that described above for the R component.

**Purification of Proteins**

In the following procedures, all operations were carried out at approximately 5°C. Centrifugations were performed using a Sorval centrifuge with GSA head for 60 to 90 min at 14,000 g. During chromatography, fractions were collected automatically. Solutions were concentrated by pressure dialysis under argon with the use of a Diaflow apparatus (Amicon) with indicated membranes.

**M Component**

Frozen cells (064 g, wet weight) of E. coli K-12 were suspended in 5 volumes of 0.05 M KPB and lysed with a Branson Sonifier, model S-125, at a setting of 4 for two 5-min periods. During sonication the temperature was kept below 8°C. The homogenate was centrifuged, and to the supernatant (34.5 g of protein) was added dropwise, with stirring, 250 ml of 2% protamine sulfate (adjusted to pH 7.5 with 1 N KOH). After being stirred for 30 min, the mixture was centrifuged, the residue discarded. Solid ammonium sulfate was added slowly, with stirring, to the supernatant (313 g per liter, 50% saturation). The suspension was then stirred for an additional 30 min and centrifuged. The supernatant was saved for isolation of the R or F components, as described below. The precipitate, which contained the M component, was dissolved in 0.14 M KPB and dialyzed against three changes of the same buffer.

In the following steps, AdoMet-chloride was added to all solutions at a final concentration of 1 μM. The dialyzed solution from the 0 to 50% ammonium sulfate precipitate was applied to a column (4 × 25.5 cm) of DEAE-cellulose. The column was washed with 0.14 M KPB, followed by gradient elution with 1 liter of 0.15 M KPB in the mixing chamber and 1 liter of 0.45 M KPB in the reservoir. The flow rate was 34 ml per hour, and 16.2-ml fractions were collected. Fractions containing the M component (tubes 19 to 72) were combined and concentrated by pressure dialysis (PM-10 membrane). The resulting solution (about 9 ml) was applied to a column (2.5 × 96 cm) of Bio-Gel P-200. Elution was performed with 0.14 M KPB/0.2 M KCl (flow rate 8.2 ml per hour, 5.4-ml fractions). Fractions containing the M component (tubes 34 to 41) were combined and diluted with an equal volume of 0.2 M KCl. This solution was applied to a column (2.5 × 37 cm) of hydroxyapatite. Gradient elution was performed (0.065 M KPB/0.2 M KCl → 0.085 M KPB/0.2 M KCl, 200 ml each; flow rate 52 ml per hour; 11.8-ml fractions). Fractions containing the M component (tubes 72 to 84) were combined and concentrated (about 5 ml) with the use of pressure dialysis (PM-10 membrane). Nine volumes of 0.15 M KPB were added,
and the solution was again subjected to pressure dialysis; this procedure (dilution and dialysis) was repeated. The solution was applied to a column (2.8 X 18.3 cm) of DEAE-Sephadex. Gradient elution was performed (0.02 M KPB → 0.39 M KPB, 250 ml each; flow rate, 17.2 ml per hour; 5.1 ml fractions). Fractions 54 to 60, which contained M component of the highest specific activity, were combined and concentrated (to about 3 ml) by pressure dialysis. In Fractions 61 to 70 the M component had a lower specific activity and a spectrum which indicated the presence of B12a.

**R Component**

The supernatant from a 0 to 50% ammonium sulfate step in the procedure for purification of M component was treated with additional solid ammonium sulfate (258 g per liter; 85% saturation). The resulting solution was centrifuged, dialyzed against 0.02 M KPB, and dialedyzed against the same buffer. The resulting solution (8.6 g of protein) was concentrated by pressure dialysis (UM-10 membrane). The concentrated solution (about 40 ml) was divided into two equal portions, each of which was filtered through a column (4 X 93.5 cm) of Sephadex G-75 (0.1 M KPB; flow rate, 25 ml per hour; 11.6-ml fractions). Fractions containing the R component (tubes 51 to 65) from each column were combined, precipitated with ammonium sulfate (85% saturation), and dialyzed against 0.04 M KPB. A solution (95 ml) was then applied to a column (3.8 × 43 cm) of DEAE-Sephadex. Two successive gradient elutions were performed: (a) 0.065 M KPB → 0.2 M KPB, 500 ml each; flow rate, 22 ml per hour; 8.7-ml fractions (tubes 44 to 47) which contained material of the highest specific activity were collected. The elution profile is shown in Fig. 1. Fractions containing R component (tubes 81 to 99) were combined, precipitated with ammonium sulfate (85% saturation), and dialyzed against 0.05 M KPB. This solution was applied to a column (1.85 × 29.6 cm) of hydroxyapatite, and gradient elution was performed (0.055 M KPB → 0.1 M KPB, 500 ml each; flow rate, 22 ml per hour; 8.7-ml fractions). The elution profile is shown in Fig. 2. Fractions containing R component were combined (tubes 44 to 67) and dialyzed against 0.04 M KPB. The solution (95 ml) was then applied to a column (1.2 × 21.5 cm) of hydroxyapatite, and gradient elution was performed (0.065 M KPB → 0.2 M KPB, 500 ml each; flow rate, 22 ml per hour; 8.7-ml fractions). The elution profile is shown in Fig. 2. Fractions containing R component (tubes 44 to 67) were combined and concentrated (to about 3 ml) by pressure dialysis (UM-2 membrane). KPB concentration was adjusted to about 0.05 M by addition of water, and the solution was rechromatographed on a hydroxyapatite column (1.2 × 21.5 cm). Gradient elution was performed (0.070 M KPB → 0.095 M KPB, 150 ml each; flow rate, 10.5 ml per hour; 3.5-ml fractions). Fractions (36 to 44) which contained material of the highest specific activity were combined and concentrated to about 2 ml by means of pressure dialysis (UM-2 membrane). Fractions 44 were treated with additional solid ammonium sulfate (258 g per liter; 85% saturation). Protein and flavoprotein were measured by absorbance at 280 and 465 nm, respectively. Assay for F activity was performed by addition of water, and the solution was rechromatographed on hydroxyapatite. Measurements were performed as in Fig. 1, except that 0.005-ml aliquots were used for the DPNH-cytochrome c reductase activity.

**F Component**

A 50 to 85% ammonium sulfate precipitate (prepared as described above for the R component) was dissolved in 150 ml of 0.28 M KPB and dialyzed against the same buffer. The resulting solution, containing 0.15 g of protein, was applied to a column (2.7 × 26 cm) of DEAE-cellulose. The column was washed with 300 ml of 0.28 M KPB; this procedure removed most of the colored components but left a yellow band at the top of the column and a brown band further down the column. Elution was then continued with a linear gradient (0.28 M KPB → 0.28 M KPB/0.6 M KCl, 500 ml each; flow rate, 43 ml per hour; 11.4-ml fractions). Protein and flavoprotein were measured by absorbance at 280 and 405 nm, respectively. Assay for F activity was performed by means of the procedure described above. The brown band was eluted first (Fractions 29 to 34). Fractions containing the yellow F component (tubes 44 to 53) were combined, diluted with an equal volume of water, and then concentrated by chromatography on a column (1.3 × 8.4 cm) of DEAE-cellulose. Elution was performed using 100 ml of 0.01 M KPB/0.7 M KCl, and the eluate containing the F component (collected visually) was concentrated to about 2 ml by pressure dialysis (UM-2 membrane). This solution was applied to a column (2.4 × 97 cm) of Sephadex G-50. Elution was performed with 0.28 M KPB (flow rate 28 ml per hour; 6.1-ml fractions). The F component, which appeared in tubes 36 to 42, was concentrated by chromatography on DEAE-cellulose, as described above, dialyzed against 0.02 M KPB/0.2 M KCl, and chromatographed on a column (1.85 × 22.7 cm) of hydroxyapatite. Gradient elution was performed (0.02 M KPB/0.2 M KCl → 0.12 M KPB/0.2 M KCl, 500 ml each; flow rate, 32 ml per hour; 8.6-ml fractions). The elution profile is shown in Fig. 3. Fractions 41 to 47, which contained M component of the highest specific activity and a spectrum which indicated the presence of B12a, were combined, precipitated with ammonium sulfate (85% saturation), and dialyzed against 0.01 M KPB.

**RESULTS**

**Activation of Methionine Synthetase by TPNH-dependent Reducing System**—In agreement with the results of previous investigations (1–5), the M component isolated by the present procedure was unable to catalyze Reaction 1 in the absence of a reducing system (Fig. 4). Addition of a chemical reducing system such as dithioerythritol plus B12a activated the methionine synthetase, and under these conditions product formation was proportional to the concentration of M. The specific activity of M, calculated from these data, was 29 μmoles of methionine synthesized per 15 min per mg of protein, or 1.9 μmoles per min per mg. The activity of M was increased considerably when the
Components of methionine-synthesizing system

Assay System A containing TPNH (or, where indicated, DPNH), 0.42 μg of M, 0.2 μg of R, and 0.2 μg of F. M component was dialyzed against 10⁻² M KPB under a continuous stream of argon in order to remove endogenous AdoMet.

Table I

| Omissions or substitutions | Methionine | nmol |
|----------------------------|------------|------|
| None                       | 13.8       |      |
| M                          | 0.0        |      |
| R                          | 0.94       |      |
| F                          | 0.49       |      |
| TPNH                       | 0.99       |      |
| DPNH replacing TPNH        | 13.9       |      |
| Homocysteine               | 0.49       |      |
| AdoMet                     | 0.54       |      |

Fig. 3. Chromatography of F component on hydroxylapatite. Protein (●) was determined as A₂₈₀. Flavoprotein (△) was measured by absorbance at 465 nm. Assay for F activity (○) was performed on 0.5-μl aliquots by means of the procedure described under “Experimental Procedures” (TPNH, 0.42 μg of M, and 0.6 μg of R; volume, 0.125 ml). Results are expressed as nanomoles of methionine per 15 min.

Fig. 4. Activation of methionine synthetase by chemical and enzymatic systems. The activity of M protein was measured in the presence of the chemical (Assay B) or the enzymatic (Assay A) systems described under “Experimental Procedures.” The enzymatic system contained TPNH, 0.4 μg of R, and 0.6 μg of F.

Fig. 5. Concentration dependence of R and F in enzymatic activating system. Top panel, Assay system A containing TPNH, 0.84 μg of M, 1.0 μg of F, and R varied as indicated. Lower panel, the same, except that 0.8 μg of R was present, and F varied as indicated.

A component study for methionine synthesis supported by the enzymatic reducing system is summarized in Table I. The following points are apparent: (a) Omission of any of the oxidation-reduction components (R, F, or TPNH) caused the activity of M to decrease markedly. The residual activities were due, in part, to the presence in the reaction mixture of homocysteine and R₃,1₅, which together provide a weak chemical reducing system. (b) Omission of other components of the assay system, such as adenosylmethionine and homocysteine, also produced a marked diminution of activity, verifying that the characteristics of methionine synthesis are not changed when M is activated by the enzymatic, rather than a chemical, reducing system. (c) Replacement of TPNH by DPNH resulted in no loss of activity, provided that the concentration of the latter nucleotide was increased sufficiently to compensate for its higher Kₘ value.

The concentration dependence of R and F for activation of the methionine synthetase is illustrated in Fig. 5. In each instance the amount of M was fixed, TPNH and one of the flavoproteins were present in excess, and the remaining component was varied as indicated. Under the conditions of these experiments, maximum activity was obtained with approximately 0.2 and 0.1 μg of the R and F components, respectively. In the 0.25-ml assay mixture, these amounts corresponded to FAD and FMN concentrations of 0.030 and 0.070 μM.

Purification and Properties of R Component—The ability of the R and F components to support methionine synthesis (cf. Fig. 5) was used to monitor these proteins during their purification. Because this activation is a catalytic rather than stoichiometric process, it was necessary to define arbitrarily the activity of the limiting component (R or F) in terms of methionine synthesized by a given amount of M component. The cytochrome c re-

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ductase activity of the R component also provided an independent (and absolute) measure of its specific activity. The parallelism between the results of the two assays for R component activity during the various steps in the purification procedure is shown in Table II. Although TPNH is more efficient than DPNH in both the R-dependent reduction of cytochrome c and in the enzymatic activation of methionine synthetase, DPNH was used for routine assays.

The 50 to 85% ammonium sulfate fraction of the cell-free extract served as the starting material for purification of the R component. A sequence of steps, described under "Experimental Procedures," separated R from other flavoproteins and led to a homogeneous preparation. During purification, care had to be taken not to confuse the R component with other cytochrome c reductases which also appeared in the elution profiles (cf. Fig. 1). It should be noted that none of these other cytochrome c reductases can substitute for R in the activation of the M component.

The final chromatographic step in the purification of the R component was characterized by a somewhat asymmetric elution profile with respect to the catalytic activities and protein. The highest specific activities and homogeneous protein (see below) were found in Fractions 38 to 44. The data in Table II indicate that the enzyme in these fractions was purified about 300- to 360-fold as judged, respectively, by the cytochrome c and methionine synthetase assays. Additional enzyme having a slightly lower specific activity was found in Fractions 34 to 37 and 45 to 54. The over-all yield of R component in Fractions 34 to 54 was about 11%. This reasonably good recovery is attributable to the stability of R during purification. It is, likewise, very stable to storage; full activity was retained after 10 months at -20°C under argon.

Homogeneity of the R component was demonstrated by electrophoresis on polyacrylamide (Fig. 6). Prior to protein staining, the material was visible as a green band at $R_f = 0.62$.

The molecular weight of the R component was determined by two procedures. Gel filtration through a standardized column of Sephadex G-75 gave a value of 26,500, which was in very good agreement with that of 27,500 from sodium dodecyl sulfate polyacrylamide electrophoresis. Based upon these results, 27,000 was chosen as the best value for the molecular weight of the R component.

The absorbance spectrum of the R component at neutral pH (Fig. 7) is unusual for a flavoprotein. Maxima occur at 274, 400, and 456 nm, with a shoulder at 480 nm. The data (Fig. 7) is unusual for a flavoprotein. Maxima occur at 274, 400, and 456 nm, with a shoulder at 480 nm; millimolar extinction coefficients calculated from the data in Fig. 7 and a molecular weight of 27,000 are 48.5, 7.4, 7.1, and 6.0, respectively, for these peaks. Heat denaturation (6 min at 97°C) of R released the flavin, and the spectrum of this chromophore is normal, i.e. maxima at 375 and 450 nm. Thin layer and paper chromatography identified this flavin as FAD. From the data in Fig. 7, and choosing 11.3 as the millimolar extinction coefficient for FAD (27), the FAD content was calculated to be 0.89 mole per mole of protein.

**Fig. 6.** Electrophoresis of M, R, and F components on polyacrylamide. The general procedure for separating and staining the proteins is given under "Experimental Procedures." A 20-μg quantity of each protein was applied to the gels.

**TABLE II**

| Step | Volume | Protein | Cytochrome c reductase | Activation of methionine synthetase |
|------|--------|---------|------------------------|-------------------------------------|
|      | ml     | mg      | Specific activity | Total units | Recovery | Specific activity | Total units | Recovery |
| 1. Cell-free extract | 6,180 | 33,400 | 0.760 | 26,800 | 100 | — | — |
| 2. Ammonium sulfate (50-85%) | 268 | 8,600 | 0.114 | 976 | — | 0.178 | 1,530 | 100 |
| 3. Sephadex G-75 | 95.0 | 1,810 | 0.292 | 538 | 54.1 | 0.568 | 1,630 | 67.3 |
| 4. DEAE-Sephadex | 39.5 | 139 | 1.122 | 294 | 19.8 | 2.09 | 475 | 31.0 |
| 5. First hydroxylapatite | 20.0 | 6.72 | 19.5 | 131 | 19.4 | 42.5 | 286 | 18.7 |
| 6. Second hydroxylapatite | 1.1 | 0.610 | 31.2 | 19.0 | 3.2 | 64.0 | 39.0 | 2.6 |
| Fractions 34-37 | 2.2 | 1.51 | 33.8 | 51.0 | 5.2 | 65.4 | 98.7 | 6.5 |
| Fractions 38-44 | 1.1 | 0.709 | 25.2 | 17.8 | 1.8 | 50.1 | 35.4 | 2.3 |

*See under "Experimental Procedures.""
on the column. Activity in the methionine synthetase system coincided with flavoprotein absorbance measured at 465 nm. The final step, chromatography on hydroxylapatite, resolved the F component into two peaks. The initial peak (Fractions 41 to 47) in Fig. 3 contained material of higher specific activity and most of the total activity. The data in Table III indicate that the enzyme in these fractions is about 250-fold purified and that the recovery is about 10%. If enzyme in the second peak (Fractions 48 to 54) is also included, the overall recovery is about 14%. Like the R component, the F component is quite stable during purification and storage (e.g. full activity is retained after 10 months at -20°C).

Homogeneity of the F component was also demonstrated by electrophoresis on polyacrylamide (Fig. 6). At pH 8.3, the F component migrated very rapidly and overlapped the marker dye ($R_f = 1.0$); this behavior is consistent with its acidic nature and low molecular weight. The F component could be detected visually on the gels as a brown band (the result of mixing the flavoprotein with the marker dye).

The molecular weight of the F component was determined by the procedures used for the R component. Gel filtration through a standard column of Sephadex G-50 and electrophoresis on sodium dodecyl sulfate polyacrylamide gave values of 19,900 and 19,500, respectively; 19,400 was taken as the best value for the molecular weight of the F component.

The absorbance spectrum of the F component at neutral pH (Fig. 8) is that of a typical flavoprotein. Millimolar extinction coefficients calculated from the data in Fig. 8 and a molecular weight of 19,400 are 53.2 at 274 nm, 7.17 at 369 nm, and 8.42 at 465 nm. Heat denaturation of F released the flavin chromophore whose spectrum is also shown in Fig. 8. Thin layer and paper chromatography identified the flavin as FMN. From the data whose spectrum is also shown in Fig. 8. Thin layer and paper chromatography identified the flavin as FMN.

**Purification and Properties of M Component—Activity of the M component was measured most conveniently during purification by Assay B (see under "Experimental Procedures") in which activation was achieved by dithioerythritol plus B$_{12}$. This chemical reducing system also aids in the removal of oxygen which may remain after flushing with argon. It should be noted that B$_{12}$ is not being used to convert apoenzyme of the M component to the holoenzyme since replacement of B$_{12}$ by FMNH$_2$ leads to no reduction in methionine synthetase activity.

Purification of the M component was achieved by the procedure described under "Experimental Procedures." In the final step, i.e. chromatography on DEAE-Sephadex, selected fractions (54 to 60) yielded a homogeneous preparation of M as judged by several criteria (see below). At this stage, the enzyme had been purified about 100-fold and was recovered in an over-all yield of about 3% (Table IV).

The loss of activity observed during purification of the M component was consistent with its instability, particularly when present in dilute solution, during storage. Maximum stability was obtained by freezing more concentrated solutions (4 to 10 mg per ml) of the protein and storing them at -20°C under argon; under these conditions, full activity was retained even after 1 year. If deoxygenation was incomplete, however, a gradual loss of activity, paralleled by an increase in absorbance of the preparation at 357 nm, was observed. AdoMet, which had been shown previously to protect the enzyme during ultracentrifugation or gel filtration (28), was found to accomplish the same purpose during purification and was included (at 1 m), therefore, in all solutions after Step 2 in the procedure. In Steps 3 to 6 of the procedure, dilute solutions of the enzyme were concentrated by pressure dialysis rather than by ammonium sulfate precipitation, since the latter was attended by an apparent oxidation of the B$_{12}$ chromophore. A similar lability, induced by exposure to ammonium sulfate, has been reported for a B$_{12}$-containing protein from Clostridium thermoaceticum (29).

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**Table III**

**Purification of F component**

| Step | Volume | Protein | Specific activity | Total units | Recovery |
|------|--------|---------|------------------|-------------|----------|
| 1. Cell-free extract$^a$ | 34,440 | 42,900 | 6.210 | 25.1 | 450 | 9.7 |
| 2. Ammonium sulfate | 207 | 9,150 | 4.69 | 37,000 | 100 |
| 3. DEAE-cellulose | 114 | 37.6 | 450 | 16,900 | 45.7 |
| 4. Sephadex G-50 | 41 | 20.9 | 524 | 10,900 | 29.5 |
| 5. Hydroxylapatite | Fractions 1-47 | 12.6 | 3.51 | 4,030 | 9.7 |
| Fractions 47-54 | 6.3 | 4.5 | 329 | 1,500 | 4.1 |

$^a$ See under "Experimental Procedures." Assay System A with TPNH, 0.94 μg of M (purified through Step 6), and 1.2 μg of R (purified through Step 5); volume, 0.25 ml.

$^b$ From 570 g of cells.

$^c$ Not determined.

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**Table IV**

**Purification of M component**

| Step | Volume | Protein | Specific activity | Total units | Recovery |
|------|--------|---------|------------------|-------------|----------|
| 1. Cell-free extract | 6,210 | 34,500 | 0.261 | 9,000 | 100 |
| 2. Ammonium sulfate | 359 | 12,700 | 0.373 | 4,730 | 52.7 |
| 3. DEAE-cellulose | 376 | 1,880 | 0.855 | 1,610 | 17.9 |
| 4. Bio-Gel P-200 | 88 | 406 | 3.29 | 1,340 | 14.9 |
| 5. Hydroxylapatite | 169 | 105 | 5.93 | 622 | 6.9 |
| DEAE-Sephadex | 2.9 | 12.3 | 25.1 | 308 | 3.4 |

$^a$ Assay System B (see under "Experimental Procedures").

$^b$ From 664 g of cells.
Homogeneity of the M component, following Step 6 in the purification procedure, was demonstrated by electrophoresis on polyacrylamide (Fig. 6). Even when larger amounts of protein (about 50 μg) were applied to the gel, only the single band at Rf = 0.41 was seen. This was visible, prior to gel protein staining, by its brown-orange color.

The molecular weight of the M component was determined by three different methods: (a) filtration through Bio-Gel P-200, and comparison of its position in the elution profile against those of various standard proteins, gave a value of 185,000; (b) electrophoresis on sodium dodecyl sulfate polyacrylamide, and comparison of its relative mobility with those of standard proteins, yielded a very similar value, viz. 187,000; and (c) conversion of the chromophore to the dicyano derivative, assuming millimolar extinction coefficients of 10.3 at 591 nm and 31.8 at 367 nm for the latter (averages of three previously reported values (30-32)), gave a slightly lower value, viz. 175,000. In view of some uncertainties inherent in the latter procedure, the first two methods were considered to be more reliable, and 186,000 was taken as the best value for the molecular weight of the M component.

The absorbance spectrum of the M component at neutral pH is shown in Fig. 9. Principal maxima occur at 278 and 474 nm with a small peak at 404 nm and a shoulder at 312 nm. The following millimolar extinction coefficients were calculated from the spectrum of the holoenzyme (Fig. 9) using a molecular weight of 186,000: 194 at 278 nm, 30.1 at 312 nm, and 11.0 at 474 nm (ratio 17.6:2.74:1.00). When the M component was treated with KCN (final concentration 0.75 mM) at pH 12.5, even in the absence of light, the spectrum of the solution changed to that of the dicyano derivative, assuming millimolar extinction coefficients of 10.3 at 591 nm and 31.8 at 367 nm for the latter (averages of three previously reported values (30-32)), giving a slightly lower value, viz. 175,000. In view of some uncertainties inherent in the latter procedure, the first two methods were considered to be more reliable, and 186,000 was taken as the best value for the molecular weight of the M component.

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These diverse observations emphasize the ability of methionine synthetase to utilize a variety of enzymatic, as well as chemical, reducing systems. However, even in these enzymatic systems, relatively large amounts of the oxidation-reduction proteins were required. Maximum stimulation of methionine synthetase was achieved, for example, when the flavoproteins were present in amounts corresponding to flavin concentrations of 10 to 20 μM. This is not much lower than the concentration (50 to 60 μM) of free, reduced flavin that would have sufficed. The present investigation was undertaken, therefore, to search for a more specific and efficient enzymatic reducing system. Fractionation of E. coli K-12 extracts by various techniques disclosed the presence of an apparently new system consisting of two separate flavoproteins which, when supplemented with TPNH (or higher concentrations of DPNH), supported methionine synthetase with a high degree of efficiency.

The R component has an unusual absorbance spectrum for a flavoprotein, viz. an anomalous band at 400 nm (Fig. 7). However, when the prosthetic group was released by heat denaturation, it showed a typical flavin spectrum (Fig. 7) and was identified chromatographically as FAD. The anomalous 400-nm band thus appears to be due to interaction of the FAD with some group on the R protein.

The F component has the characteristics of a flavodoxin (34), viz. low molecular weight, FMN as the prosthetic group, and acidic nature as evidenced by tight binding to anion exchangers and rapid mobility during electrophoresis. Vetter and Knappe (35) have reported that E. coli contains a flavodoxin whose molecular weight was found to be 13,500 to 14,500 by sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid analysis, and 19,000 to 21,000 by gel filtration and sucrose gradient centrifugation. The molecular weight found for the F component in this investigation (19,400) compares with the higher value reported by Vetter and Knappe.

The strongly acidic properties of the F component caused some difficulties in the initial purification of this protein. When preparations were chromatographed on DEAE-cellulose, elution with KPB alone removed most of the protein but none of the F activity; it was found that the latter could be eluted by including 0.6 M KC1 in the buffer. The acidic nature of F is probably also responsible for its association with other proteins. Thus, in a trial experiment, passage of a 50 to 85% ammonium sulfate fraction...
through Sephadex G-75 resulted in the appearance of F activity in the R fraction, as well as in fractions having both higher and lower molecular weights. For this reason, procedures were developed (see under "Experimental Procedures") for purifying R and F from separate batches of the 50 to 85% ammonium sulfate fraction. In the case of the F component, the key procedure (Step 3) involved chromatography of this fraction on DEAE-
cellulose that had been equilibrated with 0.28 M KBP; this resulted in a 100-fold purification of F in a single step. The final step in the purification of F (chromatography on hydroxylapatite) led to the appearance in the eluate of one major flavoprotein peak accompanied by a minor peak. The material in both peaks had the same visible absorbance spectrum and small molecular weight (as judged by their inability to be separated by filtration through Sephadex G-50). Although each fraction was effective in the activation of methionine synthetase, the material in the minor peak had a lower specific activity due to contamination by inert protein.

B_{12}-containing methionine synthetases isolated previously from several strains of E. coli (1–3) have varied considerably with respect to purity, specific activity, molecular weight, absorbance spectrum, and B_{12} content. In the present investigation, the synthetase was found to be homogeneous by electrophoresis on polyacrylamide (Fig. 6) and sodium dodecyl sulfate polyacryl-
amide (not shown). Its specific activity was 1.9 μmoles per min per mg of protein when the dithioerythritol plus B_{12a} activating system was used. The TPNH-dependent enzymatic system al-

dowed M to function at a higher specific activity, viz. 3.8 μmoles per min per mg. This latter value, taken in conjunction with the molecular weight, corresponds to an apparent turnover num-
ber at 37° of 706 moles of methionine synthesized per min per mole of enzyme. A maximum turnover number of 1100 can be obtained when the measurement is carried out after an initial lag phase of the reaction has been completed.*

The molecular weight of the M component, determined by gel filtration (183,000), was nearly identical with that obtained by sodium dodecyl sulfate electrophoresis (187,000); it is of interest that such a large protein apparently consists of a single polypep-
tide chain. Lower molecular weights, ranging from 125,000 to 150,000, have been reported for other E. coli methionine synthetases (2, 3, 12, 36). Rüdiger (37), however, obtained a higher value of 255,000 for a preparation in which a milder procedure was used; this larger value may be due to an association of the synthetase with oxidation-reduction proteins similar to those described in this paper.

The absorbance maxima at 474 and 312 nm in the spectrum of the M component (Fig. 9) suggests that the chromophore is B_{12}; if so, interaction with the protein must confer a stability toward air oxidation upon the B_{12} moiety. Unlike preparations from other strains of E. coli (2, 3), the present enzyme has little abso-

rption in the 350 to 360 nm region. As measured by conver-
sion of the chromophore to diethyldiB_{12}, the B_{12} content of the present enzyme is 1.06 mole per mole of protein (mol wt 186,000). In contrast, the amount of enzyme-bound corrinoid was estimated to be 0.35 mole per mole of protein by Taylor and Weiss-
bach (2), and 0.51 to 0.59 mole per mole by Stavrianopoulos and Jaenicke (3) in their respective preparations of the enzyme from E. coli B. Although the latter preparation appeared to be homoge-

nous in the ultracentrifuge, it was not examined electro-

phoretically. Recently, Taylor (38) has utilized preparative electrophoresis to obtain a sample of the synthetase that was un-
contaminated by other proteins, but the B_{12} content and the spectrum of the enzyme were not given.

From this investigation, it is clear that activation of the E. coli K 12 methionine synthetase can be achieved in a highly efficient manner by an enzymatic system consisting of TPNH operating in conjunction with the two flavoproteins, R and F; evidence will be presented in a subsequent communication that transfer of reducing equivalents occurs in the following order: TPNH → R → F → M. However, in view of the fact that a variety of chemical and enzymatic systems can also activate methionine synthetase, the question may be raised as to whether the TPNH-R-F system is the "physiological" activator. It is possible, of course, that methionine synthetase does not require "activation" in the cell, but if it does, this process probably could not be accom-

plished by chemical reductants which would be present only at relatively low concentrations. Enzymatic activation is thus the more likely alternative. This is supported by the efficiency of the present system which appears to be due to the ability of F to interact more favorably than other flavoproteins with the M component. It is perhaps also significant that, based upon calcu-

lations from data in Tables II to IV, the three protein components (R, F, and M) are present in approximately equimolar amounts in the cell-free extract of E. coli K 12 and that maximum activa-
tion is obtained when all three protein components are present at approximately equimolar concentrations in the assay system (cf. Fig. 5).

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