Cobalamin-dependent methionine synthase from Escherichia coli B: Electron paramagnetic resonance spectra of the inactive form and the active methylated form of the enzyme

ARTICLE *in* BIOCHEMISTRY · DECEMBER 1988

Impact Factor: 3.02 · DOI: 10.1021/bi00422a025 · Source: PubMed

Cobalamin-Dependent Methionine Synthase from *Escherichia coli* B: Electron Paramagnetic Resonance Spectra of the Inactive Form and the Active Methylated Form of the Enzyme†

Verna Frasca, Ruma V. Banerjee, William R. Dunham, Richard H. Sands, and Rowena G. Matthews*

Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Received June 1, 1988; Revised Manuscript Received July 18, 1988

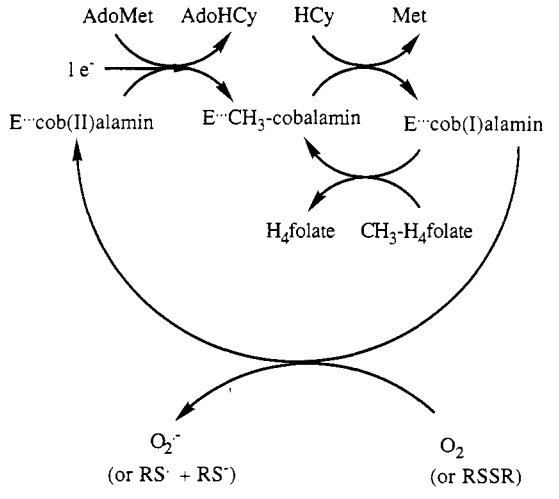
ABSTRACT: Cobalamin-dependent methionine synthase (5-methyltetrahydrofolate–homocysteine methyltransferase, EC 2.1.1.13) has been isolated from *Escherichia coli* B in homogeneous form. The enzyme is isolated in an inactive form with the visible absorbance properties of cob(II)alamin. The inactive enzyme exhibits an electron paramagnetic resonance (EPR) spectrum at 38 K that is characteristic of cob(II)alamin at acid pH, where the protonated dimethylbenzimidazole substituent is not coordinated with the cobalt nucleus (base-off cobalamin). An additional, variable component of the EPR spectrum of the inactive enzyme has the characteristics of a cob(III)alamin–superoxide complex. Previous work by others [Taylor, R. T., & Weissbach, H. (1969) *Arch. Biochem. Biophys. 129*, 745–766. Fujii, K., & Huennekens, F. M. (1979) in *Biochemical Aspects of Nutrition* (Yagi, K., Ed.) pp 173–183, Japan Scientific Societies, Tokyo] has demonstrated that the enzyme can be activated by reductive methylation using adenosylmethionine as the methyl donor. We present data indicating that the conversion of inactive to methylated enzyme is correlated with the disappearance of the EPR spectrum as expected for the conversion of paramagnetic cob(II)alamin to diamagnetic methylcobalamin. When the methyl group is transferred from the methylated enzyme to homocysteine under aerobic conditions, cob(II)alamin/cob(III)alamin–superoxide enzyme is regenerated as indicated by the return of the visible absorbance properties of the initially isolated enzyme and partial return of the EPR spectrum. Our enzyme preparations contain copper in ~1:1 stoichiometry with cobalt as determined by atomic absorption spectroscopy. This copper is EPR silent in the enzyme as isolated and in methylated enzyme, but it can be detected as a cupric–EDTA complex following aerobic denaturation of the enzyme in the presence of mersalyl, 6 M urea, and EDTA.

Cobalamin-dependent methionine synthase (5-methyltetrahydrofolate–homocysteine methyltransferase, EC 2.1.1.13) catalyzes the final reaction in the de novo biosynthesis of methionine (eq 1). As isolated from *Escherichia coli*, the

$$CH_3\text{-}H_4folate + HCy \rightarrow H_4folate + Met \quad (1)$$

enzyme is inactive and has the visible absorbance properties of cob(II)alamin (Taylor & Weissbach, 1967a; Fujii & Huennekens, 1974). There is an absolute requirement for catalytic amounts of AdoMet[1] (Mangum & Scrimgeour, 1962; Foster et al., 1964) and a reducing system (Guest et al., 1962) for the formation and maintenance of active enzyme during in vitro turnover. Fujii and Huennekens (1974) have shown that two flavoproteins can be isolated from cell extracts of *E. coli* that activate methionine synthase in the presence of AdoMet and NADPH. These two flavoproteins are designated the R protein, which shows NADPH–flavodoxin oxidoreductase activity, and the F protein, which has the physical properties of a flavodoxin. Anaerobic incubation of methionine synthase with these two proteins and an excess of NADPH results in changes in the visible absorbance spectrum of the enzyme that are consistent with the conversion of the enzyme-bound cobalamin prosthetic group from cob(II)alamin to methylcobalamin (Fujii & Huennekens, 1979). These authors also report that methylcobalamin is released from the activated enzyme on treatment with hot ethanol/water (80:20).

Scheme I: Interconversion of the Forms of Methionine Synthase



Studies by Taylor and Weissbach (1969a,b) have established that the methyl group of [*methyl*-^{14}C]AdoMet is transferred to the cobalamin prosthetic group during the activation process and that this transfer occurs only in the presence of a reducing

† This work was supported in part by U.S. Public Health Service Grants GM 24908 (R.G.M.) and GM 32785 (R.H.S.) from the National Institute of General Medical Sciences.

* Address correspondence to this author at the Biophysics Research Division.

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; CH_3-H_4folate, 5-methyltetrahydrofolate; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; $FMNH_2$, reduced flavin mononucleotide; FPLC, fast protein liquid chromatography; HCy, homocysteine; KPB, potassium phosphate buffer.

0006-2960/88/0427-8458$01.50/0

system. Taylor and Weissbach (1969b) have also shown that the methylated enzyme, freed of unbound AdoMet by gel filtration, can catalyze 8–10 turnovers aerobically and 800–1000 turnovers anaerobically in the absence of the reducing system.

These studies have led to the formulation of catalysis shown in Scheme I. Spectrophotometric evidence for the formation of cob(I)alamin during demethylation of methylated enzyme by homocysteine was obtained by Taylor and Hanna (1970) and by Fujii and Huennekens (1979). These workers also provided evidence for the remethylation of the cob(I)alamin enzyme by CH_3-H_4folate (Taylor & Weissbach, 1969a; Fujii & Huennekens, 1979). There is net retention of configuration of a chiral methyl group after enzyme-catalyzed methyl transfer from CH_3-H_4folate to methionine (Zydowsky et al., 1986), which supports the double-displacement model for methyl transfer. The cob(I)alamin form of the enzyme is reported to be extremely labile aerobically (Taylor & Weissbach, 1969b) and also to decay rapidly under anaerobic conditions (Fujii & Huennekens, 1979) to form the inactive cob(II)alamin enzyme. This inactivation is presumably due to transfer of electrons to oxygen under aerobic conditions. Under anaerobic conditions inactivation may be due to the reduction of disulfide contaminants of the thiols present in in vitro assays (Hogenkamp et al., 1985).

Identification of the inactive form of the enzyme-bound prosthetic group as cob(II)alamin rested primarily on its visible absorbance properties and on the fact that the treatment of the enzyme with alkaline cyanide resulted in the formation of a dicyanocobalamin, as expected for a cobalamin that lacks an alkyl ligand. Taylor and Weissbach (1967a) failed to observe an EPR spectrum of the inactive enzyme at 25 °C, although cob(II)alamin should be paramagnetic (Hogenkamp et al., 1963). These studies were initiated to clarify the oxidation state of the inactive enzyme and have shown that it is indeed a cob(II)alamin. Since the EPR spectrum of cob(II)alamin reflects the conformation and environment of the prosthetic group, considerable information about the enzyme active site may be obtained.

Experimental Procedures

Materials. The following materials were purchased from Sigma: AdoMet (chloride and iodide salts), aquocobalamin, bovine serum albumin, L-homocysteine thiolactone, (6-*ambo*)-CH_3-H_4folate (barium salt), NADPH, phenylmethanesulfonyl fluoride, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, and *p*-(hydroxymercuri)benzoate agarose. Bio-Gel HTP, Bio-Rex 70, Dowex AG1-X8, and protein dye reagent were purchased from Bio-Rad. DEAE-Sephadex A-50 and Sephadex G-25 were from Pharmacia, and Amicon supplied Matrex Gel Red A. Dithiothreitol was purchased from Boehringer Mannheim. Safety-Solve scintillation fluid was from Research Products International. (6-*ambo*)-5-[*methyl*-^{14}C]CH_3-H_4folate (barium salt, 58 mCi/mmol) was purchased from Amersham. DEAE-52 was from Whatman.

E. coli B cell paste was obtained from Grain Processing Corp., Muscatine, IA; the cells were grown aerobically in Kornberg minimal medium supplemented with 0.15 μM cyanocobalamin and were harvested at $^3/_4$ log phase. HCy was formed from its thiolactone as described by Hatch et al. (1961). [*adenosyl*-U-^{14}C]AdoMet was prepared enzymatically from [U-^{14}C]ATP and methionine as described by Sumner et al. (1986). AdoMet was purified on a Bio-Rex 70 column (Sumner et al., 1986) prior to use in the formation of methylated enzyme. The R and F proteins were purified from *E. coli* B by methods adapted from those described by Fujii and Huennekens (1974), as outlined by Frasca (1986).

Enzyme Assay. Assay mixtures (1 mL) contained 100 mM potassium phosphate buffer, pH 7.2, 500 μM HCy, 125 μM (6*S*)-5-[*methyl*-^{14}C]CH_3-H_4folate (2000 dpm/nmol, added as the 6-ambo derivative, 19 μM AdoMet (iodide salt), 50 μM aquocobalamin, 25 mM dithiothreitol, and methionine synthase. The reaction was initiated by addition of CH_3-H_4folate after a 5-min preincubation of the assay mixture at 37 °C; 10 min after initiation, the reaction was terminated by heating at 98 °C for 2 min. The assay mixture was cooled on ice and then passed through a 0.5 × 6 cm column of Bio-Rad AG1-X8 (chloride form). The eluate was collected in a scintillation counting vial containing 14 mL of Safety-Solve. The reaction tube was rinsed with 2 mL of water, and the rinse was collected in the vial. All reported assay values were corrected for the counts observed in control assays from which methionine synthase was omitted. Samples were counted in a Beckman LS7500 liquid scintillation counter. One unit of methionine synthase activity catalyzed the formation of 1 μmol of methionine/min at 37 °C.

Protein Assay. Protein concentrations were determined by the Bio-Rad protein assay, based on the method of Bradford (1976); bovine serum albumin was used as the standard protein. The protein concentrations of fractions from column chromatography were estimated from their absorbance at 280 nm.

Purification of Methionine Synthase from E. coli B. All steps were performed at 4 °C unless otherwise indicated. All buffers contained potassium phosphate, pH 7.2 (KPB), at the indicated concentrations. Protein solutions were concentrated under nitrogen in an Amicon ultrafiltration cell fitted with a PM 30 membrane, and concentrated enzyme solutions were desalted by continuous diafiltration in the ultrafiltration cell by washing with 4 volumes of dialysis buffer. The flow of buffer from an Amicon RC 800 reservoir was controlled by an Amicon concentration/dialysis selector (CDS-10). Small volumes of protein were concentrated in Centricon 30 microconcentrators (Amicon).

Step 1. Preparation of Cell-Free Extract. Frozen *E. coli* paste (1000 g) was thawed overnight at 4 °C; the cells were suspended in 2 L of 50 mM KPB, and the mixture was gently stirred for 1 h to produce a smooth homogenate. Phenylmethanesulfonyl fluoride (400 mg) and tosyl-L-lysine chloromethyl ketone (20 mg) were added to the suspension. One-liter aliquots of the cells were disrupted with a Branson Sonifier, Model 185, at an output setting of 7 for four 10-min periods with a 5-min break between sonication cycles to prevent overheating the solution. The cell suspension was kept below 10 °C with an ice/salt bath, and the mixture was gently stirred during sonication. Cell debris and unbroken cells were removed from soluble protein by centrifugation at 10000*g* for 90 min in a JA-10 rotor of a Beckman J2-21 centrifuge. The pooled supernatant was stored overnight at −20 °C and then was recentrifuged at 30000*g* for 45 min in a JA-18 or JA-20 rotor to remove additional insoluble precipitate that formed overnight.

Step 2. Batch Chromatography on DEAE-cellulose. The cell-free extract was diluted with 1 volume of cold deionized water. DEAE-52, equilibrated with 50 mM KPB, was filtered to dryness and added batchwise to the protein solution; 1 g of filtered DEAE-52 was added per 1.67 units of enzyme activity. The slurry was stirred for 90 min, and the resin was filtered on a Büchner funnel to remove unbound protein. The DEAE-52 cake was resuspended in 2 L of 100 mM KPB, stirred for 30 min, and then filtered. Methionine synthase was

eluted from the DEAE-52 with two 1000-mL aliquots of 400 mM KPB after stirring for 60 min, and the eluate was separated from the DEAE-52 by filtering.

Note. In all of the steps that follow, buffer and enzyme solutions contained 1 μM AdoMet chloride to help stabilize methionine synthase activity, and all concentrated enzyme solutions were brought to 0.01% (w/v) tosyl-L-lysine chloromethyl ketone to help prevent proteolysis.

Step 3. DEAE-Sephadex Chromatography. The enzyme solution from the previous step was diluted with 1.2 volumes of cold deionized water to make the KPB concentration 180 mM. The solution was applied simultaneously to two columns of DEAE-Sephadex A-50 (2.5 × 20 cm) equilibrated with 180 mM KPB. When enzyme application was completed, the columns were rinsed with 0.8 L of 180 mM KPB, and the enzyme was eluted from each column with a 500-mL linear gradient of 180–480 mM KPB. Fractions of 5 mL were collected. The peak of enzyme activity eluted at ~300 mM KPB. Fractions containing enzyme activity were pooled, concentrated to 75 mL, and equilibrated with 25 mM KPB by dialfiltration.

Step 4. Matrex Gel Red A Chromatography. The enzyme solution from the previous step was applied to a 1.5 × 20 cm column of Matrex Gel Red A that had previously been equilibrated with 25 mM KPB. Fractions (5 mL) were collected as the protein solution was passing through the column. Methionine synthase does not bind to the resin, and enzyme activity began to elute after 10–15 mL of protein solution had been applied. The column was rinsed with 50 mL of 25 mM KPB to remove remaining methionine synthase, and enzyme-containing fractions were pooled.

Step 5. Chromatography on p-(Hydroxymercuri)benzoate Agarose. The enzyme from the previous step was brought to 50 mM in KPB, and the protein was applied to a *p*-(hydroxymercuri)benzoate agarose column (1.5 × 30 cm) previously equilibrated with 50 mM KPB. The column was rinsed with 100 mL of 50 mM KPB and then with 50 mL of the same buffer containing 20 mM dithiothreitol. Methionine synthase was eluted with a 300-mL linear gradient of 0–500 mM KCl in 50 mM KPB containing 20 mM dithiothreitol, and 3-mL fractions were collected. Enzyme activity eluted at approximately 150 mM KCl. Fractions with the highest specific activities were pooled, concentrated to about 20 mL, and equilibrated with 5 mM KPB/0.2 M KCl by diafiltration.

Step 6. Chromatography on Bio-Gel HTP. The enzyme from the previous step was applied to a 1.5 × 20 cm column of Bio-Gel HTP that had previously been equilibrated with 5 mM KPB/0.2 M KCl. The column was rinsed with 50 mL of the same buffer, and methionine synthase was eluted with a 300-mL linear gradient of 5–60 mM KPB in 0.2 M KCl. Fractions of 3 mL were collected. The fractions with the highest specific activity were pooled and equilibrated with 20 mM KPB/20% glycerol and concentrated to 1–2 mL. At this stage of purification the enzyme was routinely 60–90% homogeneous on the basis of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had a specific activity of 5–11 units/mg of protein. Enzyme activity was quite stable on prolonged storage in this buffer at −70 °C.

Step 7. Chromatography on Aminohexyl-Sepharose 4B. Enzyme was brought to 0.2 M in KCl was applied to a 1 × 20 cm column of aminohexyl-Sepharose 4B previously equilibrated with 20 mM KPB/0.2 M KCl. The enzyme was eluted with a 300-mL linear gradient of 0.2–1.0 M KCl in 20 mM KPB; the peak of enzyme activity eluted at ~0.6 M KCl. The purest fractions were pooled, concentrated to 1.5–2 mL, and equilibrated with 20 mM KPB/20% glycerol for storage at −70 °C.

Modified Purification of the Enzyme Using Fast Protein Liquid Chromatography. For later studies the purification procedure was modified as follows: After the completion of step 5, the enzyme was loaded onto a Pharmacia Mono Q 10/10 FPLC column, washed with 40 mL of 50 mM KPB, then with 40 mL of 95 mM KPB, and eluted with a 160-mL linear gradient of 95–500 mM KPB. Fractions containing methionine synthase activity were pooled, equilibrated with 50 mM KPB, and concentrated. They were then rechromatographed under the same conditions. Prior to long-term storage at −70 °C the active fractions were equilibrated with 50 mM KPB/20% glycerol and concentrated.

Regeneration of p-(Hydroxymercuri)benzoate Agarose. This resin can be used repeatedly with proper regeneration. Noguchi et al. (1978) reported a regeneration method using salt and 2-mercaptoethanol. Our regeneration method was adapted from their technique with advice from Sigma; the resin was rinsed with 100 mL of 100 mM cysteine in 50 mM KPB followed by 100 mL of 10 mM 2-mercaptoethanol/0.5 M KCl in 50 mM KPB, and finally with 100 mL of 50 mM KPB.

Determination of Metal Content of Purified Methionine Synthase. The cobalamin concentration of enzyme preparations was estimated from their absorbance at 470 nm by using an extinction coefficient of 11 000 M^{-1} cm^{-1} (Fujii & Huennekens, 1974). The cobalt, iron, copper, and manganese contents of enzyme preparations were also measured by flameless atomic absorption spectroscopy, performed in the laboratory of Dr. Ronald Rossmann (Great Lakes Research Division, The University of Michigan) by James Barres. The analyses were performed on a Perkin-Elmer Model 5000 spectrometer with graphite furnace, using the method of standard additions. All values are corrected for the metal content of the buffer in which the sample was dissolved.

Formation of Methylated Methionine Synthase. The method of preparation was adapted from one described by Fujii and Huennekens (1979). Purified, concentrated methionine synthase (~40 nmol in 1 mL of 20 mM KPB/20% glycerol) was placed in an anaerobic cuvette, and the following components were added: R protein (0.1 mol/mol of methionine synthase), F protein (0.05 mol/mol of methionine synthase), and AdoMet (40 μM). Oxygen was removed from the cuvette with eight cycles of evacuation followed by nitrogen equilibration using an anaerobic train described previously (Williams et al., 1979). A solution of NADPH (equilibrated with nitrogen and containing 10 mol NADPH/mol of methionine synthase) was added to the enzyme solution from a gastight Hamilton syringe after an initial visible absorbance spectrum was recorded at 30 °C on a Cary 118C spectrophotometer. Spectra were recorded at intervals after NADPH addition. A control experiment was also performed in which 20 mM KPB replaced NADPH. When spectrophotometric changes were completed, unbound AdoMet and the flavoproteins were separated from methionine synthase by concentrating the protein to 0.5 mL and rinsing with five 1.5-mL aliquots of 20 mM KPB/20% glycerol in a Centricon 30 microconcentrator.

Electron Paramagnetic Resonance Spectroscopy. Samples for EPR spectroscopy were placed in 4-mm (o.d.) EPR tubes and frozen in liquid nitrogen. They were stored under liquid nitrogen until spectra were recorded. EPR spectra were recorded on an X-band Varian E-line spectrometer with a custom-made gas-phase helium transfer line. Exact settings are shown in the figure legends. Signal averaging was performed on multiple transients using a Tracor-Northern NS-570

Table I: Purification of Cobalamin-Dependent Methionine Synthase from *E. coli* B

step	total units act. (μmol/min)	protein[a] (mg)	sp. act. (units/mg)	yield (%)	purification (x-fold)
(1) cell-free extract[b]	800	59800	0.013	100	1
(2) batch DEAE-cellulose chromatography	629	7500	0.084	79	6
(3) DEAE-Sephadex chromatography	270	740	0.37	34	29
(4) Amicon Matrex Gel Red chromatography	270	370	0.73	34	56
(5) *p*-(hydroxymercuri)benzoate agarose chromatography	130	43	3.0	16	230
(6) hydroxyapatite chromatography	61	12	5.2	8	400
(7) aminohexyl-Sepharose 4B chromatography	29	2.5	11.6	4	890

[a] Determined by Bio-Rad protein assay with bovine serum albumin as the standard. [b] From 1280 g of *E. coli* cell paste.

signal averager. The digitized data were transferred to a microcomputer for spin quantitation, which consisted of comparison of the second definite integral of the sample spectra with that of a cupric perchlorate standard (1.0 mM $CuSO_4$, 10 mM HCl, 2M $NaClO_4$). This standard was chosen for the simplicity of its EPR spectrum relative to those of other cupric compounds (Hagen, 1982). We did not correct the integrals for the differences in g values between samples and standards (Aasa & Vanngard, 1975) because the discrepancy between the estimated g values of the samples and those of the standard were small ($\bar{g}_{av}$ for Cu^{2+}, 2.19; $\sim\bar{g}_{av}$ for methionine synthase, 2.19).

Determination of the Apparent Molecular Weight of Native Methionine Synthase by Gel Filtration. The apparent molecular weight of methionine synthase was determined by chromatography on a Sepharose 12 FPLC column and comparison of the elution volume with those of thyroglobulin, ferritin, aldolase, and ovalbumin. Protein samples were equilibrated with 50 mM KPB, pH 7.2, containing 0.15M KCl and chromatographed at a flow rate of 0.74 mL/min. The eluant was monitored at 280 nm with an LKB Uvicord S recorder equipped with a 2.5-mm, 8-μL HPLC flow cell to determine the elution volume. In the case of methionine synthase, the activity of the eluate was shown to coincide with a symmetrical peak eluting at 11.7 mL.

Results

Purification of Cobalamin-Dependent Methionine Synthase. The enzyme has been purified to homogeneity from *E. coli* B. The purification scheme is summarized in Table I. It is similar in several respects to the procedures used for partial purification of the enzyme from *E. coli* B by Taylor and Weissbach (1967a) and for purification to homogeneity of the enzyme from *E. coli* K12 (Fujii & Huennekens, 1974), both of which use anion-exchange chromatography on DEAE-cellulose and chromatography on hydroxyapatite. As additional steps, we use a negative purification step involving passage of the enzyme over a column of Matrex Gel Red A, and we also perform chromatography of the enzyme on *p*-(hydroxymercuri)benzoate agarose and on aminohexyl-Sepharose 4B. Using this method of purification, we routinely isolate enzyme that is 50–100% homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and our yields range from 4 to 30% of the initial activity in crude extracts. Figure 1 shows a photograph of a slab gel electrophoresed in the presence of sodium dodecyl sulfate and stained with Coomassie blue. Aliquots of enzyme containing 2×10^{-3} units of activity from steps 3–7 of the purification were applied to the gel lanes. After step 7, the enzyme in this preparation appeared homogeneous and had an apparent subunit molecular weight of 133 000. As judged from the nearly constant intensity of the 133-kDa band in each lane, the activity of the enzyme does not decrease significantly during purification. The specific activity of homogeneous preparations of enzyme was 9.3–11.7 μmol min^{-1} (mg of protein)$^{-1}$. Enzyme purified by FPLC had lower specific activity (~6 units/mg of protein) and was not homogeneous, but showed identical spectral properties by both visible absorbance and EPR spectroscopy. The typical increase in specific activity associated with FPLC chromatography after step 5 was 2–4-fold, with a 60% step yield.

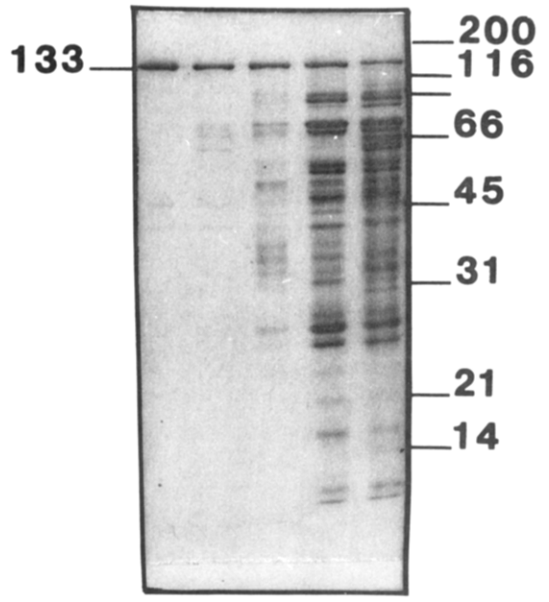


FIGURE 1: Polyacrylamide gel electrophoresis of methionine synthase at different stages during its purification. Samples of methionine synthase were diafiltered with 50 mM KPB and then denatured by boiling for 2 min in a solution of 5 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8. Samples were applied to a discontinuous gel (4% polyacrylamide stacking gel, 10% polyacrylamide running gel). After electrophoresis, the gel was stained with Coomassie blue, destained, and dried to filter paper. Each lane contained 2×10^{-3} units of methionine synthase from the indicated step: [lane 1 (far right)] after chromatography on DEAE-Sephadex A-50; (lane 2) after chromatography on Amicon Matrex Gel Red A; (lane 3) after chromatography on *p*-(hydroxymercuri)benzoate agarose; (lane 4) after chromatography on hydroxyapatite; (lane 5) after chromatography on aminohexyl-Sepharose 4B. The molecular weight markers were myosin (200 000), β-galactosidase (116 000), phosphorylase B (92 500), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

Physical Properties of the Enzyme. The native enzyme has an apparent molecular weight of 153 000 as determined by gel filtration on a calibrated Sepharose 12 FPLC column. This number is in good agreement with the subunit molecular weight of 133 000 determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and suggests that the native enzyme is monomeric. The molecular weight of the native enzyme is also in good agreement with the value reported for the *E. coli* B enzyme of 137 000 (Taylor & Weissbach, 1967). On two-dimensional gels, run as described by Bloch et al. (1980), the protein migrates at 95 × 120, migrating in the horizontal dimension with an apparent

Table II: Physical Properties of Methionine Synthase Preparations

prepn	sp. act.	cobalamin content (μM) from A_{470}	cobalamin content (μM) by atomic absorption	ratio	Fe/Co[c]	Cu/Co[c]
172	3.7	28.0	29.1	0.96	0.30	ND
175	11.6	5.9	5.4	1.09	0.44	1.57
176[a]	7.1	7.9	10.1	0.78	0.95	1.00
177	11.7	8.1	10.4	0.78	0.3	1.1
178	8.4	28.6	38.5	0.74	ND	1.73
179	6.3	36.7	48.2	0.74	ND	0.74
182[b]	4.7	11.4	8.5	1.34	ND	0.89
av				0.92 ± 0.23		1.17 ± 0.39

[a] Fe^{2+}, 100 μM, added to cell-free extract before purification. [b] EDTA, 0.3 mM, added to all buffers used during purification. [c] Cobalamin content on the basis of atomic absorption.

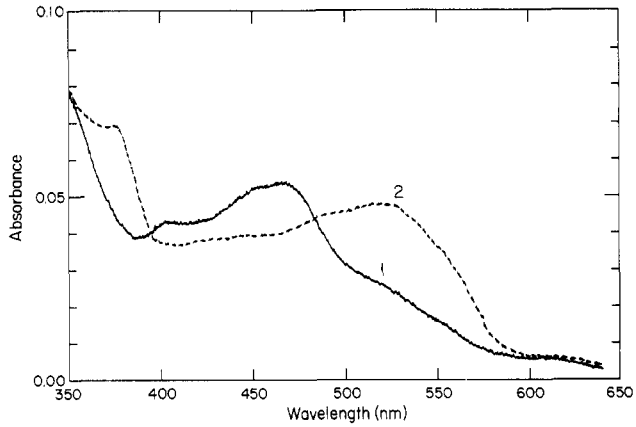


FIGURE 2: Absorbance spectra of methionine synthase as isolated and after methylation: (1) Spectrum of purified enzyme (5.2 μM in 20 mM KPB, 20% glycerol); (2) corrected spectrum of methylated enzyme. The enzyme was methylated as described under Experimental Procedures and then rinsed with 10 mL of 20 mM KPB/20% glycerol and concentrated to 1 mL in a Centricon 30 microconcentrator. The spectrum shown was corrected to adjust for a 15% protein loss during rinsing and concentration.

isoelectric point of ~5.2 and in the vertical dimension with an apparent molecular weight of 137 000. The alphanumeric assigned to this protein is C137 (Pedersen et al., 1978).

The cobalt content of several enzyme preparations has been measured by atomic absorption spectroscopy, and the results are shown in Table II. Our enzyme preparations exhibit a visible absorbance spectrum with a maximum at 470 nm, and measurement of the cobalamin content using an extinction coefficient at 470 nm of 11 000 M^{-1} cm^{-1} (Fujii & Huennekens, 1974) gives values in good agreement with the cobalt content determined by atomic absorption spectroscopy. The ratio of cobalt content determined by absorption at 470 nm to that determined by atomic absorption was 0.92 ± 0.23 (n = 7). Enzyme preparations were also analyzed for iron, copper, and manganese by atomic absorption spectroscopy, and the results of iron and copper analyses are also shown in Table II. No manganese was detected in our enzyme preparations.

Methylation of Methionine Synthase in the Presence of AdoMet and a Reducing System. Figure 2 shows results obtained before and after the methylation of a homogeneous preparation of enzyme with high (11.6) specific activity. The observed absorbance changes are very similar to those reported by Fujii and Huennekens (1979) and are consistent with the conversion of a cob(II)alamin prosthetic group to methyl cob(III)alamin with a coordinated (base-on) dimethylbenzimidazole substituent.

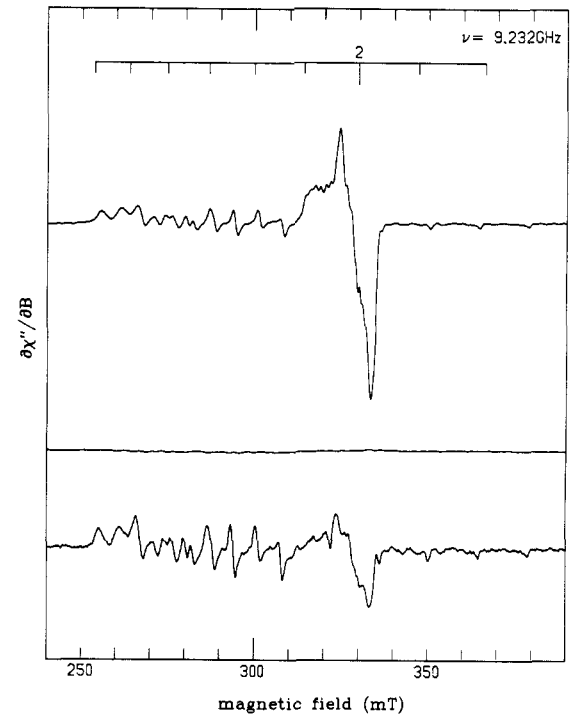


FIGURE 3: EPR spectra of enzyme as isolated, methylated enzyme, and enzyme after demethylation: (Upper trace) Enzyme as isolated (spectrum adjusted to 23 μM concentration; actual enzyme concentration, 43 μM). Spectra (16 scans) were recorded at 40 K, 10-mW microwave power, and 1-mT modulation amplitude with a resolution of 2048 points. (Middle trace) 23 μM methylated enzyme, with spectra recorded under the same conditions. (Lower trace) 23 μM demethylated enzyme (produced by addition of 4 mM homocysteine to the enzyme solution and incubation for 1 h at 37 °C). Spectra (8 scans) were recorded under the same conditions.

Electron Paramagnetic Resonance Spectroscopy of Methionine Synthase. Figure 3 shows the electron paramagnetic resonance spectrum of methionine synthase before and after reductive methylation. The upper trace represents an aliquot of enzyme as isolated. The cobalt content of the enzyme determined spectrophotometrically was 43 μM, and the EPR spin quantitation gave a value of 42 μM. The lower trace represents an aliquot of the same enzyme taken at the completion of methylation and subjected to EPR spectroscopy. No EPR signal could be detected in this sample. The methylated enzyme was then incubated with homocysteine under aerobic conditions, until the visible absorbance spectrum had returned to that characteristic of the enzyme as isolated. The EPR spectrum of this demethylated enzyme differs qualitatively from that of the original enzyme in that the feature at $g \sim 2.0$ is greatly reduced and the overall amplitude of the signal is diminished. Spin quantitation of the EPR signal gave a value of 12 μM, although the cobalamin content was 23 μM as determined by spectrophotometry. This discrepancy in the EPR quantitation of demethylated enzyme is presently not understood. Since the proportion of the feature at $g \sim 2.0$ differs in the spectra of the enzyme as isolated and the demethylated enzyme, a difference spectrum can be generated that contains only the features not associated with the $g = 2.0$ signal. This difference spectrum is shown in Figure 4.

The amount of the $g \sim 2.0$ signal seen varies from one preparation to another, and some preparations exhibit only this signal, as shown in Figure 5. This spectrum is characteristic of cob(III)alamin superoxide (Bayston et al., 1969; Cockle et al., 1970; Pilbrow, 1982), and spin quantitation gave a value of 15 μM, which agreed well with the cobalamin content estimated from absorbance at 470 nm (14.1 μM). This

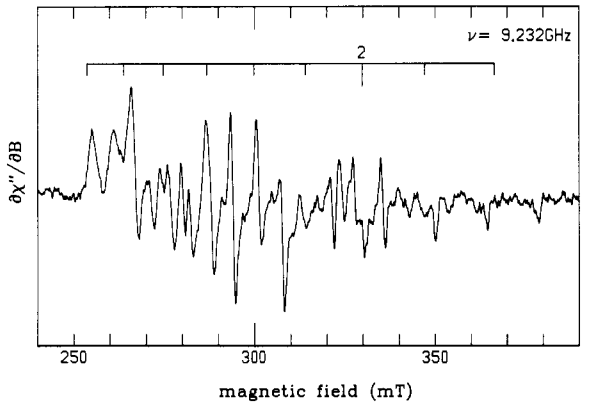


FIGURE 4: Difference EPR spectrum of the cob(II)alamin component of methionine synthase. This spectrum was generated by subtracting $^2/_{10}$ of the spectrum of enzyme as isolated (upper trace, Figure 3) from the spectrum of the demethylated enzyme.

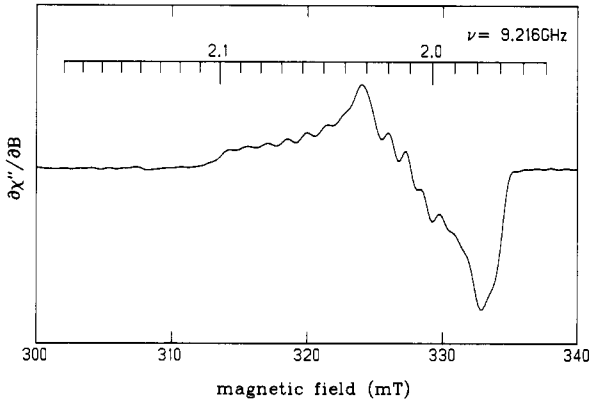


FIGURE 5: EPR spectrum of a methionine synthase preparation with the properties of a cob(III)alamin–superoxide complex. Spectra (4 scans with a 512-point resolution) of the enzyme (14.1 μM) were recorded under the same conditions as those of the samples in Figure 3.

enzyme preparation was active [specific activity of 5.4 μmol min^{-1} (mg of protein)$^{-1}$] and could be methylated.

The EPR spectrum of preparations of methylated enzyme gave no indication of the presence of paramagnetic Cu^{2+}, even though atomic absorbance indicated the presence of copper. Incubation of a preparation of methylated enzyme containing 0.74 mol copper/mol of cobalamin with ferricyanide (100 μM) for 30 min at 37 °C prior to EPR spectroscopy did not affect the EPR spectrum. We then incubated methylated enzyme with buffer containing 6 M urea, 2.5 mM mersalyl, and 0.3 mM EDTA for 48 h at 25 °C in order to denature the protein and form a copper–EDTA complex. The EPR spectrum of methylated enzyme, after denaturation, is shown in Figure 6. Spin quantitation of the copper signal gave a spin concentration of 13 μM, showing a 1:2 stoichiometry with the estimated cobalamin concentration after denaturation (25.4 μM), and several other preparations have yielded substoichiometric levels of copper as determined by EPR spectroscopy after denaturation. Other enzyme preparations have yielded copper in stoichiometric (1:1) ratios with cobalamin after denaturation. Given the difficulty of denaturing the enzyme to release the copper (appearance of the copper EPR signal requires many hours of incubation in the denaturing buffer), the measurement of lower levels of copper by EPR spectroscopy than by atomic absorption may not be surprising.

Discussion

Cobalamin-dependent methionine synthase from *E. coli* B has been purified to homogeneity by a new method. The

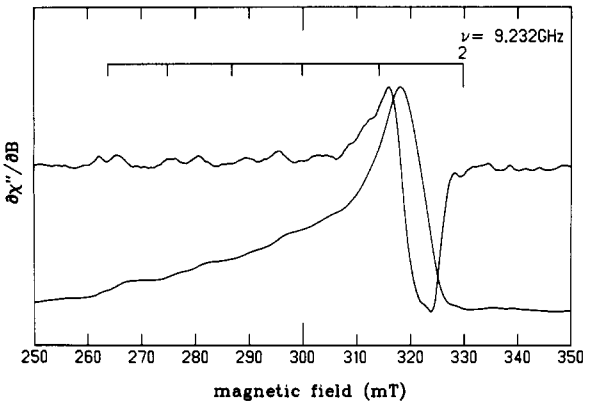


FIGURE 6: EPR spectrum of a denatured methylated methionine synthase preparation. Enzyme was brought to 6 M in urea, 2.5 mM in mersalyl, and 0.3 mM in EDTA and incubated at 25 °C for 48 h. Spectra (4 scans with a 512-point resolution) were recorded at 40 K with a microwave power of 2 mW and a modulation amplitude of 1 mT. The lower trace shows the second definite integral of the EPR spectrum and was used to determine the spin concentration of copper in the denatured enzyme.

specific activity of the homogeneous enzyme, measured aerobically with a dithiothreitol/aquocobalamin reducing system, varies from 9.3 to 11.7 μmol min^{-1} (mg of protein)$^{-1}$. This range of specific activities corresponds to a range of turnover numbers at 37 °C of 1240–1560 min^{-1} when a molecular weight per cobalamin of 133 000 was used. These values are substantially higher than the turnover number of 310 reported for the homogeneous enzyme from *E. coli* K12 when an anaerobic assay with a dithiothreitol/aquocobalamin reducing system was used (Fujii & Huennekens, 1974) but compare closely with the turnover number of 1100 min^{-1} calculated for the homogeneous enzyme from *E. coli* K12 by using the R/F/NADPH reducing system after a 10-min lag phase had elapsed (Fujii et al., 1977). The enzyme prepared by Taylor and Weissbach (1967a) from *E. coli* B was estimated to be 33% pure from its cobalamin content, and from their data we calculate a turnover number for homogeneous enzyme of ~ 200 min^{-1}, using an aerobic assay with a 2-mercaptoethanol and cyanocobalamin reducing system, and of 800 min^{-1} with an anaerobic assay using an $FMNH_2$ reducing system. We do not observe a substantial difference in the activity of our protein preparations measured aerobically with dithiothreitol/aquocobalamin or anaerobically with R/F/NADPH.

Our preparations of enzyme contain an average of 0.35 mol of Fe/mol of Co and 1.17 mol of Cu/mol of Co. The copper appears to be extremely tightly bound and is not removed on dialysis of the protein against buffers containing EDTA. If the cell extract is prepared by sonication in buffers containing EDTA and all purification steps are conducted with buffers containing EDTA, the enzyme as isolated contains 0.89 mol of Cu/mol of Co. Copper also is not displaced if ferrous iron is added to the cell extract, and the purified protein then contains 1 mol of Cu and 1 mol of Fe per mole of cobalamin. We are not aware of any previous reports in which the metal content of the bacterial cobalamin-dependent methionine synthase has been examined, but the homogeneous enzyme from human placenta is reported to contain 2 mol of Fe/mol of Co (Utley et al., 1985). Thus far, we have been unable to determine whether the metal ion is required for catalysis. The copper is bound extremely tightly to the enzyme, and we have only been able to release it under conditions where the protein is irreversibly denatured. We are presently unable to perform the definitive experiment of showing loss of enzyme activity when the metal ion is removed and regain of activity on re-

constitution. We might expect that chelation of a metal ion involved in catalysis would lead to inhibition, but in the case of methionine synthase interpretation of such experiments is complicated by the fact that some of the best chelators of copper, such as cyanide, also form strong coordination complexes with the cobalt in cobalamin.

Although an average of 1 Cu/subunit is present in these enzyme preparations, we have not been able to detect an EPR signal due to Cu^{2+} in the native enzyme. Perhaps the most likely explanation is that copper is present in a diamagnetic redox state, e.g. Cu^{+} or Cu^{3+}. Alternatively, the copper may be spin paired with an unidentified paramagnetic component of the enzyme preparation. However, the cobalamin, which is EPR detectable in native enzyme as isolated, cannot be involved in spin pairing. We are presently in the final stages of sequencing the cloned gene for cobalamin-dependent methionine synthase from *E. coli* and hope to be able to identify the copper binding site by analogy with those seen in other proteins (Banerjee et al., 1988). We also plan to determine the oxidation state of the bound copper by EXAFS in collaboration with Dr. James Penner-Hahn.

In conjunction with our measurements of the EPR spectra of preparations of unmethylated and methylated enzyme, we have examined the reaction in which enzyme is methylated in the presence of the R/F/NADPH reducing system and AdoMet. In agreement with the published studies of Fujii and Huennekens (1979) we see changes in the visible absorbance spectra that are consistent with the conversion of the cob(II)alamin prosthetic group to a base-on methylcobalamin prosthetic group with an absorbance maximum at 520 nm.

EPR spectroscopy of methionine synthase, in the form in which it was isolated, shows the presence of a paramagnetic ion in 1:1 stoichiometry with the cobalamin content of the protein. The spectrum we observe shows strong similarities to published EPR spectra of base-off cobalamin, cobinamide, and porphyrin complexes containing cobalt(II) (Pilbrow, 1982, and references cited therein; de Bolfo et al., 1976; Bayston et al., 1970). Base-on and base-off cobalamin EPR spectra can readily be distinguished by the pattern and spacing of their high-field peaks, which result from the interaction of the unpaired electron in the dz^2 orbital with the cobalt nucleus and, in the case of a cobalamin in which the dimethylbenzimidazole substituent is coordinated to the cobalt nucleus in the lower axial position, with the nitrogen of dimethylbenzimidazole. Interaction of the electron with the cobalt nucleus (spin $^7/_2$) results in hyperfine splitting to produce eight equally spaced peaks in the EPR spectrum. Superhyperfine splitting by the coordinated dimethylbenzimidazole, if it occurs, results in further splitting of each of the eight peaks to form a triplet. Thus, base-on cobalamins show spectra with a series of high-field triplets with ~10.5-mT spacing, while base-off cobalamins and cobinamides that lack a nitrogenous axial ligand show spectra with a series of high-field singlets with ~14–16-mT spacing (Bayston et al., 1970; Pilbrow, 1982). The EPR spectra shown in Figure 4 contain a series of high-field singlet peaks with a 14.1-mT spacing, and the g_z value associated with this octet of peaks is estimated to be 2.004. Hill et al. (1971) have reported g_z values of 2.004–2.006 for cob(II)alamins at pH 0, where the dimethylbenzimidazole substituent is protonated, and $g_{x,y}$ values of 2.4–2.6 and 2.2. The $g_\perp$ regions of our spectra are more difficult to interpret, and simulation will be required to obtain accurate estimates of the g values and hyperfine interaction constants. In the absence of simulation an exact value for $g_\perp$ cannot be determined, but it appears to be located around $g = 2.3$, in good agreement with the range of g values observed for base-off cobalamins generated by acidification. Due to the uncertainty of the exact g values and the fact that any corrections would be small, we have not attempted to correct our spin quantitations for discrepancies between the g values of the standard ($g_x = 2.073$, $g_y = 2.086$, $g_z = 2.397$; $g_{av} = 2.189$) and those of the enzyme-bound cobalamin.

As noted under Results, some of our preparations of enzyme exhibit exclusively an EPR spectrum characteristic of a cob(III)alamin–superoxide complex, and all preparations exhibit at least a contribution due to this complex. We do not detect differences in the visible absorbance spectra of enzyme preparations that differ in the content of cob(III)alamin–superoxide complex. Since the enzyme is isolated aerobically, it is remarkable that the cob(II)alamin prosthetic group is so resistant to air oxidation or even to formation of a cob(III)alamin–superoxide complex during isolation. Cob(II)alamin itself is oxidized by molecular oxygen in aqueous solutions. Since the enzyme-bound cob(III)alamin–superoxide complex disappears during reductive methylation of the enzyme, we assume that this form of the enzyme can be reduced by the NADPH/R/F reducing system.

A methylated enzyme sample was denatured to obtain the copper–EDTA spectrum seen in Figure 6. Any residual unmethylated cobalamin is rapidly oxidized to cob(III)alamin on denaturation and is EPR silent. Inspection of the spectrum of native methylated enzyme revealed no apparent feature in the region of 315–325 mT, where cupric ion should absorb energy, so the copper in methylated enzyme is not detectable by EPR spectroscopy. Since the cobalt in methylcobalamin is diamagnetic and there is only ~1 Cu/mol of cobalamin, the copper is presumably not present as an unpaired Cu^{2+}. A small feature in the 315–325-mT region can be discerned in the spectrum of the enzyme as isolated, but this feature can account for no more than 1–2% of the total spin.

If copper is present as Cu^{+} in the native enzyme, its stability to oxidation by either oxygen or ferricyanide is an unusual feature, which must result from its interaction with the protein. The 600–800-nm region of the visible absorbance spectrum of native enzyme is flat (data not shown) and shows no evidence of the charge-transfer complexes characteristic of enzymes containing blue copper(II) or copper(III) (Malkin & Malmstrom, 1970; Hamilton et al., 1978).

Our observation of a base-off spectrum for the cob(II)alamin form of methionine synthase at 38 K adds to the body of evidence suggesting that this enzyme undergoes facile interconversion between base-off and base-on conformations. An accessible base-off conformation for propylated holoenzyme was first suggested by Hogenkamp (1968) on the basis of the observed shift of the visible absorbance maximum to *shorter* wavelengths associated with propylation (Taylor & Weissbach, 1967b). Studies of the methylated holoenzyme formed by methyl transfer from CH_3-H_4folate showed that this form of the enzyme exhibits temperature-dependent visible absorbance properties, shifting from a spectrum with a peak at 520 nm at 18 °C to one with a peak at 455 at 40 °C (Fujii & Huennekens, 1979). These changes are consistent with a temperature-dependent shift in the equilibrium between base-on and base-off conformers.

While this work was in progress, Ragsdale and co-workers (1987) published the results of EPR spectroscopy of the corrinoid protein involved in synthesis of acetyl CoA by *Clostridium thermoaceticum*. The corrinoid protein accepts the methyl group of methyltetrahydrofolate in a reaction catalyzed by methyltransferase. This methyl group is sub-

sequently transferred from the corrinoid protein to carbon monoxide dehydrogenase. The corrinoid protein thus catalyzes a reaction that is very similar to the methionine synthase methyl-transfer sequence. The authors found that the enzyme, as isolated, exhibited the visible absorbance spectrum of cob(II)alamin and exhibited an EPR spectrum characteristic of a base-off cobalamin, with large cobalt hyperfine splitting (142-mT spacing) and no superhyperfine interactions. In contrast to methionine synthase, methylation of the corrinoid protein leads to a visible absorbance spectrum with a maximum at 457, characteristic of a base-off methylcobalamin. In both these enzymes, methylation requires reductive activation, and a cob(I)alamin form of the enzyme is the species that accepts the methyl group from methyltetrahydrofolate.

Enzymatic stabilization of the base-off conformer of cob(II)alamin at neutral pH should facilitate reduction of the enzyme to cob(I)alamin. Model studies have established that the reduction of base-off cob(II)alamin to cob(I)alamin is more favorable both thermodynamically and kinetically (Lexa & Saveant, 1976, 1983). Base-off alkylcobalamins are profoundly stabilized toward homolytic fission of the carbon–cobalt bond (Schrauzer & Grate, 1981; Halpern et al., 1984; Marzilli et al., 1985) and provide increased accessibility of the axial alkyl group to nucleophilic displacement (Hogenkamp et al., 1987). The alternate formation and heterolytic cleavage of the carbon–cobalt bond associated with the methyl transfers catalyzed by cobalamin-dependent methionine synthase may be facilitated by the interconversion of base-on and base-off conformers of the enzyme.

Registry No. EC 2.1.1.13, 9033-23-2; AdoMet, 29908-03-0; NADPH, 53-57-6; copper, 7440-50-8; iron, 7439-89-6.

References

Aasa, R., & Vanngard, T. (1975) *J. Magn. Reson. 19*, 308–315.

Banerjee, R. V., Frasca, V., Johnston, N. L., Ballou, D. P., Datta, P., & Matthews, R. G. (1988) *Biochemistry 27*, 3101.

Bayston, J. H., King, N. K., Looney, F. D., & Winfield, M. E. (1969) *J. Am. Chem. Soc. 91*, 2775–2779.

Bayston, J. H., Looney, F. D., Pilbrow, J. R., & Winfield, M. E. (1970) *Biochemistry 9*, 2162–2172.

Bloch, P. L., Phillips, T. A., & Neidhardt, F. C. (1980) *J. Bacteriol. 141*, 1409–1420.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Cockle, S. A., Hill, H. A. O., & Williams, R. J. P. (1970) *Inorg. Nucl. Chem. Lett. 6*, 131–134.

Daubner, S. C., Krapf, E. C., & Matthews, R. G. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 617–620, MacMillan, London.

deBolfo, J. A., Smith, T. P., Boas, J. F., & Pilbrow, J. R. (1976) *J. Chem. Soc., Dalton Trans.* 1495–1500.

Foster, M. A., Dilworth, M. J., & Woods, D. D. (1964) *Nature (London) 201*, 39–42.

Frasca, V. (1986) Ph.D. Dissertation, The University of Michigan, Ann Arbor.

Fujii, K., & Huennekens, F. M. (1974) *J. Biol. Chem. 249*, 6745–6753.

Fujii, K., & Huennekens, F. M. (1979) in *Biochemical Aspects of Nutrition* (Yagi, K., Ed.) pp 173–183, Japan Scientific Societies, Tokyo.

Fujii, K., Galivan, J. H., & Huennekens, F. M. (1977) *Arch. Biochem. Biophys. 178*, 662–670.

Guest, J. R., Friedman, S., & Foster, M. A. (1962) *Biochem. J. 84*, 93P–94P.

Hagen, W. R. (1982) Ph.D. Dissertation, University of Amsterdam, Amsterdam.

Halpern, J., Kim, S.-H., & Leung, T. W. (1984) *J. Am. Chem. Soc. 106*, 8317–8319.

Hamilton, G. A., Adolf, P. K., de Jersey, J., DuBois, G. C., Dyrkacz, G. R., & Libby, R. D. (1978) *J. Am. Chem. Soc. 100*, 1899–1912.

Hatch, F. T., Larrabee, A. R., Cathou, R. E., & Buchanan, J. M. (1961) *J. Biol. Chem. 236*, 1095–1101.

Hill, H. A. O., Pratt, J. M., & Williams, R. J. P. (1971) *Methods Enzymol. 28*, 5–31.

Hogenkamp, H. P. C. (1968) *Annu. Rev. Biochem. 37*, 225–248.

Hogenkamp, H. P. C., Barker, H. A., & Mason, H. S. (1963) *Arch. Biochem. Biophys. 100*, 353–359.

Hogenkamp, H. P. C., Bratt, G. T., & Sun, S. (1985) *Biochemistry 24*, 6428–6432.

Hogenkamp, H. P. C., Bratt, G. T., & Kotchevar, A. T. (1987) *Biochemistry 26*, 4723–4727.

Lexa, D., & Saveant, J.-M. (1976) *J. Am. Chem. Soc. 98*, 2652–2658.

Lexa, D., & Saveant, J.-M. (1983) *Acc. Chem. Res. 16*, 235–243.

Malkin, R., & Malmstrom, B. G. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 177–239.

Mangum, J. H., & Scrimgeour, K. G. (1962) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 21*, 242.

Marzilli, L. G., Summers, M. F., Bresciani-Pahor, N., Zangrando, E., Charland, J.-P., & Randaccio, L. (1985) *J. Am. Chem. Soc. 107*, 6880–6888.

Noguchi, E., Nishikimi, M., & Yagi, K. (1978) *Anal. Biochem. 91*, 367–369.

Pedersen, S., Bloch, P. L., Reeh, S., & Neidhardt, F. C. (1978) *Cell (Cambridge, Mass.) 14*, 179–190.

Pilbrow, J. R. (1982) in B_{12} (Dolphin, D., Ed.) Vol. 1, pp 432–462, Wiley-Interscience, New York.

Pilbrow, J. R., & Winfield, M. E. (1973) *Mol. Phys. 25*, 1073–109.

Ragsdale, S. W., Lindahl, P. A., & Munck, E. (1987) *J. Biol. Chem. 262*, 14289–14297.

Schrauzer, G. N., & Grate, J. H. (1981) *J. Am. Chem. Soc. 103*, 541–546.

Sumner, J., Jencks, D. A., Khani, S., & Matthews, R. G. (1986) *J. Biol. Chem. 261*, 7697–7700.

Taylor, R. T., & Weissbach, H. (1967a) *J. Biol. Chem. 242*, 1502–1508.

Taylor, R. T., & Weissbach, H. (1967b) *J. Biol. Chem. 242*, 1509–1516.

Taylor, R. T., & Weissbach, H. (1969a) *Arch. Biochem. Biophys. 129*, 728–744.

Taylor, R. T., & Weissbach, H. (1969b) *Arch. Biochem. Biophys. 129*, 745–766.

Taylor, R. T., & Hanna, M. L. (1970) *Arch. Biochem. Biophys. 137*, 453–459.

Utley, C. S., Marcell, P. D., Allen, R. H., Antony, A. C., & Kolhouse, J. F. (1985) *J. Biol. Chem. 260*, 13656–13665.

Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62*, 185–198.

Zydowsky, T. M., Courtney, L. F., Frasca, V., Kobayashi, K., Shimizu, H., Yuen, L.-D., Matthews, R. G., Benkovic, S. J., & Floss, H. G. (1986) *J. Am. Chem. Soc. 108*, 3152–3153.