A Synthetic Module for the *metH* Gene Permits Facile Mutagenesis of the Cobalamin-Binding Region of *Escherichia coli* Methionine Synthase: Initial Characterization of Seven Mutant Proteins†

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ABSTRACT: Cobalamin-dependent methionine synthase from *Escherichia coli* is a monomeric 136 kDa protein composed of multiple functional regions. The X-ray structure of the cobalamin-binding region of methionine synthase reveals that the cofactor is sandwiched between an α-helical domain that contacts the upper face of the cobalamin and an α/β (Rossmann) domain that interacts with the lower face. An unexpected conformational change accompanies binding of the methylcobalamin cofactor. The dimethylbenzimidazole ligand to the lower axial position of the cobalt in the free cofactor is displaced by histidine 759 from the Rossmann domain [Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., & Ludwig, M. L. (1994) *Science 266*, 1669]. In order to facilitate studies of the roles of amino acid residues in the cobalamin-binding region of methionine synthase, we have constructed a synthetic module corresponding to nucleotides (nt) 1741−2668 in the *metH* gene and incorporated it into the wild-type *metH* gene. This module contains unique restriction sites at ∼80 base pair intervals and was synthesized by overlap extension of 22 synthetic oligonucleotides ranging in length from 70 to 105 nt and subsequent amplification using two sets of primers. Expression of methionine synthase from a plasmid containing the modified gene was shown to be unaffected by the introduction of the synthetic module. *E. coli* does not synthesize cobalamin, and overexpression of MetH holoenzyme requires accelerated cobalamin transport. Growth conditions are described that enable the production of holoenzyme rather than apoenzyme. We describe the construction and initial characterization of seven mutants. Four mutations (His759Gly, Asp757Glu, Asp757Asn, and Ser810Ala) alter residues in the hydrogen-bonded network His-Asp-Ser that connects the histidine ligand of the cobalt to solvent. Three mutations (Phe708Ala, Phe714Ala, and Leu715Ala) alter residues in the cap region that covers the upper face of the cobalamin. The His759Gly mutation has profound effects, essentially abolishing steady-state activity, while the Asp757, Ser810, Phe708, and Leu715 mutations lead to decreases in activity. These mutations assess the importance of individual residues in modulating cobalamin reactivity.

Cobalamin-dependent methionine synthase from *Escherichia coli* catalyzes the transfer of a methyl group from CH_3-H_4folate[1] to homocysteine, generating H_4folate and methionine. This enzyme employs a methylcobalamin cofactor that plays an essential role in the methyl transfer mechanism, being alternately demethylated by homocysteine and remethylated by CH_3-H_4folate. Demethylation of the methylcob(III)alamin cofactor during the catalytic cycle results in the formation of a cob(I)alamin prosthetic group, and this highly reduced form of cobalamin is occasionally oxidized to the inactive cob(II)alamin form of the enzyme. Return of the cob(II)alamin enzyme to the catalytic cycle requires a reductive methylation, in which the electron is supplied by reduced flavodoxin (Fujii & Huennekens, 1974) and the methyl group comes from AdoMet (Mangum & Scrimgeour, 1962). The enzyme must catalyze three different methyl transfer reactions involving different substrates and different oxidation states of the cobalamin prosthetic group [see Scheme 1 in the accompanying paper (Jarrett et al., 1996)].

Recently the X-ray structure of a fragment containing the 27 kDa cobalamin-binding region of *E. coli* methionine synthase was determined (Drennan et al., 1994a). The

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; Hcy, L-homocysteine; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria broth; CH^+═H_4folate, 5,10-methenyltetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; H_4folate, 5,6,7,8-tetrahydrofolate.

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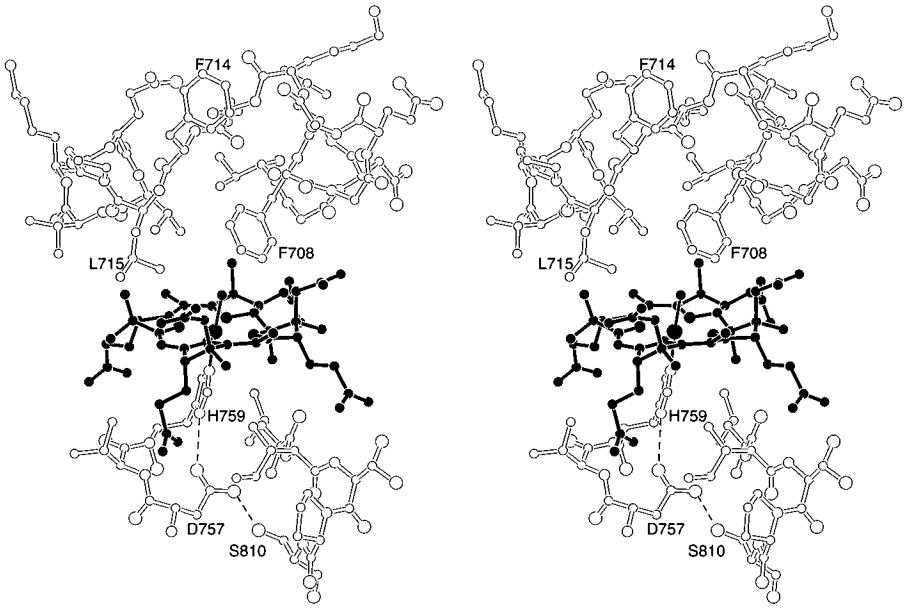

FIGURE 1: Stereoview showing the amino acid residues (open bonds) above and below the enzyme-bound methylcobalamin (closed bonds) in methionine synthase. Above the corrin ring, the sidechain of Phe708 is positioned over the methyl group of methylcobalamin, while Leu715 flanks its left side. In contrast, the side chain of Phe714 projects above the protein backbone and does not contribute to shielding of the prosthetic group from solvent. His759, the lower ligand to the cobalt, is connected by a hydrogen bond (dashed lines) to Asp757 which in turn is hydrogen bonded to Ser810. The dimethylbenzimidazole moiety of the cobalamin has been omitted for clarity.

cobalamin-binding region of methionine synthase consists of two domains: an α/β (Rossmann) domain that interacts with the lower face of the cofactor and an α-helical domain that contacts the upper face. In addition, the structure revealed an unexpected conformational change in the bound cofactor. The dimethylbenzimidazole group, which is coordinated to the cobalt in the free cofactor, is displaced from the cobalt and is replaced by a histidine contributed by the α/β domain of the protein. This histidine ligand (His759) is hydrogen bonded to an aspartate residue (Asp757), which in turn is hydrogen bonded to a serine (Ser810) (Figure 1). These residues constitute a ligand triad, and we have proposed that the triad plays an important role in modulating cobalamin reactivity (Drennan et al., 1994a). The top face of the corrin ring is covered by hydrophobic residues from the α-helical domain. This cap region forms a hydrophobic cage around the methyl group, limiting access to and from the active site, and may be important in protecting the resting methylcobalamin form of the enzyme from photolysis and from reaction with adventitious nucleophiles. Reaction of substrates with the cobalamin may require displacement of the cap region. Residues Phe708 and Leu715 contribute to the hydrophobic cage and are in van der Waals contact with the upper face of the cobalamin cofactor. (see Figure 1). The structure of the cobalamin-binding region suggests that mutations in the hydrogen-bonded ligand triad and in residues contacting the upper face of methylcobalamin might have significant effects on catalysis.

Methionine synthase is a large monomeric polypeptide of 136 kDa (Drummond et al., 1993b), consisting of functional regions that can be isolated by proteolysis of the native enzyme with trypsin (Drummond et al., 1993a). The 27 kDa cobalamin-binding region generated by tryptic digestion spans residues 644−896 of the 1227 amino acid polypeptide (Drummond et al., 1993b). Because the *metH* gene is large, few unique restriction sites are available to facilitate mutagenesis in the wild-type gene. Furthermore, the large size is incompatible with construction of an entirely synthetic gene because the distances between possible unique restriction sites would be too large to permit facile synthesis of oligonucleotides for overlap extension. Accordingly, we decided to construct a synthetic module for the cobalamin-binding region that would then be embedded within the wild-type gene using preexisting unique restriction sites. This strategy could easily be adapted to regions of other large proteins; indeed, we are currently constructing a synthetic module corresponding to the C-terminal 38 kDa portion of methionine synthase.

In considering strategies for overexpression of methionine synthase, we were faced with another complication. The cofactor is tightly bound in this enzyme, and the X-ray structure reveals that the dimethylbenzimidazole substituent of the cobalamin is deeply embedded in the Rossmann domain, suggesting that proper protein folding might require the cobalamin to be present. Aerobically grown *E. coli* do not synthesize cobalamin, and it is brought into the cell by an energy-dependent transport system (Bradbeer, 1991). Thus the expression of MetH protein must be induced under conditions that permit simultaneous transport of cobalamin at a rate sufficient to allow incorporation of cobalamin during the folding process.

In this work, we report construction of a synthetic gene module for the cobalamin-binding fragment of the methionine synthase gene. A 930 bp sequence specifying the cobalamin-binding region of methionine synthase was altered such that unique restriction sites were introduced at ~80 bp intervals without changing the final amino acid sequence and introduced into the *metH* gene. Expression of the protein using the wild-type or semisynthetic gene of methionine synthase was not affected by the nucleotide sequence changes in the semisynthetic gene. Seven mutations were chosen to probe structure-based hypotheses about the roles of residues in the His-Asp-Ser ligand triad and the hydrophobic cap. We report the initial characterization of these mutants by steady-state kinetics.

MATERIALS AND METHODS

Materials. Restriction enzymes *Bpu*1102-I, *Bsp*EI, *Bst*EII, *Nhe*I, *Sac*I, *Spe*I, *Bgl*II, *Eag*I, and *Xma*I were purchased from New England BioLabs. *Eco*RI, *Hin*dIII, *Sal*I, and *Xho*I were purchased from Life Technologies, *Mlu*I and *Acc*I were from Promega, and *Spl*I was purchased from Amersham. Low melting agarose was from FMC Bioproducts. (6-*R*,*S*)-5-methyltetrahydrofolate was from Schirks Laboratories.

DNA Oligomer Synthesis. DNA oligomers were made on an ABI model 392 DNA synthesizer (Applied Biosystems) using β-cyanoethyl phosphoramidate chemistry (Caruthers et al., 1987). The oligonucleotides were deblocked by incubating in concentrated ammonium hydroxide at 56 °C for 10−14 h and purified using Oligonucleotide Purification Cartridges (Applied Biosystems) following the manufacturer's instructions.

Subcloning of the metH Gene into Expression Vector pKK223-3. The starting point for the subcloning was the plasmid p4B6.3 (Banerjee et al., 1989), shown in Figure 2A. This plasmid incorporates a DNA fragment containing the *metH* gene and promoter region inserted between the *Eco*RI and *Sal*I sites of pGEM4B (Promega). In summary, subcloning entailed the following steps: removal of extraneous DNA both upstream and downstream of the *metH* coding sequence; incorporation of an *Eco*RI site just upstream of the start codon and a second TGA stop codon and *Hin*dIII site just downstream of the TGA stop codon at nt 3681; and removal of an internal *Hin*dIII site at nt 3147 in the *metH* gene. This modified coding sequence was then cloned into expression vector pKK223-3 (Pharmacia) between the *Eco*RI and *Hin*dIII sites of the polycloning region.

To remove ~1.1 kb of sequence downstream of the *metH* stop codon, a 556 bp fragment of DNA encompassing the region between the *Hin*dIII site and the stop codon was amplified from template p4B6.3 using Vent DNA polymerase (New England BioLabs) and primers 1 and 2. Primer 1

5′-GCGCCGAAGCTTTCTGGTAAAGCAG-3′ [primer 1]

5′-gcggcaagctttcaTCAGTCCGCGTCATACCCCAG-3′ [primer 2]

contains nt 3141−3165 of the sense strand of the *metH* gene, while primer 2 is complementary to nt 3663−3684. Nucleotides in the primers that match residues in the *metH* gene are shown in capital letters. Primer 2 incorporates an extra TGA stop codon and a *Hin*dIII site into the downstream end of the amplified sequence. The fragment generated using PCR was gel purified using the Gene Clean kit (Bio 101), digested with *Hin*dIII and ligated into the *Hin*dIII, site of pGEM4B. The resulting plasmid was designated pKF550 (Figure 2B). Plasmids p4B6.3 and pKF550 were digested with the restriction endonuclease *Acc*I. The *Acc*I-digested pKF550 was treated with calf intestinal alkaline phosphatase (Promega) for 1 h at 37 °C. The 4.9 kb *Acc*I fragment containing the upstream region of *metH* in the digest of p4B6.3 was purified from a 1% agarose gel using the Gene Clean kit, ligated into pKF550, and then transformed into strain XL1-Blue (Stratagene). A clone containing a plasmid with the insert in the correct orientation was designated XL1-Blue/pKF5.2 (Figure 2C).

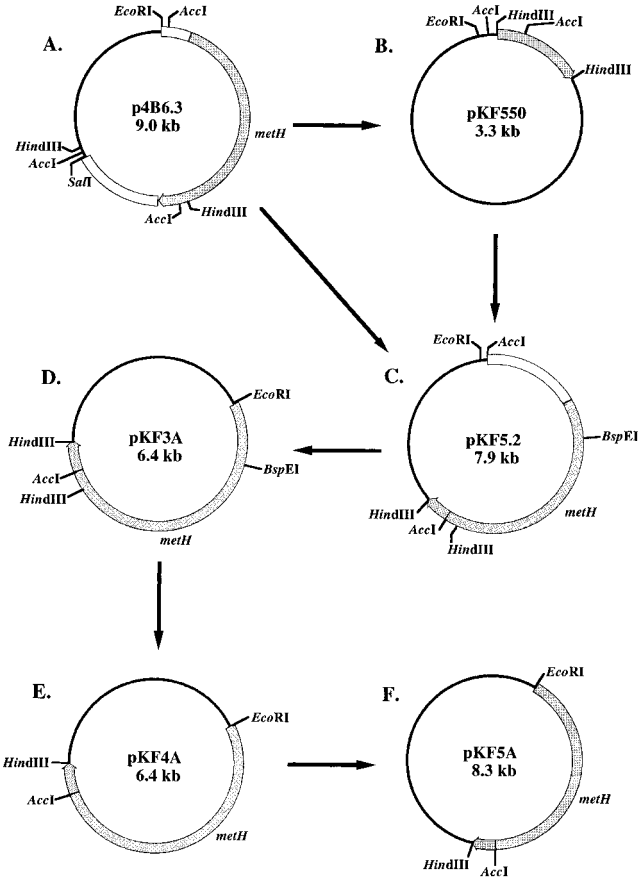

FIGURE 2: Subcloning of the *metH* gene into expression vector pKK223-3. (A) The starting point for the subcloning is p4B6.3 (Banerjee et al., 1989). (B) PCR amplification using primers 1 and 2 (see Materials and Methods) and digestion with *Hin*dIII produced a fragment extending from the *Hin*dIII site in the *metH* gene to a *Hin*dIII site introduced at the end of the coding sequence. This fragment was cloned into the *Hin*dIII site of pGEM4B to create pKF550. (C) pKF550 and p4B6.3 were digested with *Acc*I; the insert containing the upstream region of the *metH* gene from p4B6.3 was ligated into the vector fragment from pKF550 to give pKF5.2. (D) A PCR fragment extending from the start codon of *metH* to a region downstream of the *Bsp*EI site in *metH* and incorporating an *Eco*RI site upstream of the start codon was digested with *Bsp*E1 and *Eco*RI and ligated into pKF5.2 to form pKF3A. (E) The internal *Hin*dIII site in *metH* was removed by mutagenesis to form pKF4A. (F) The *metH* coding region was removed by digestion with *Eco*RI and *Hin*dIII and ligated into pKK223-3 to form pKF5A.

To remove ~1.5 kb of sequence upstream of the start codon of *metH* in p4B6.3, the polymerase chain reaction was performed with Vent DNA polymerase, p4B6.3 template DNA, and primers 3 and 4. Primer 3 contains nt 1−23 from

5′-ccgaattcagtaTGAGCAGCAAAGTGGAACAACTG-3′ [primer 3]

5′-GGATCGTTGACGTCCGGAGAAATA-3′ [primer 4]

the sense strand of *metH*, and primer 4 is complementary to nt 422−445, downstream of a *Bsp*EI restriction site in the *metH* sequence; regions corresponding to the original *metH* sequence are shown in capital letters. Primer 3 incorporates an *Eco*RI site upstream of the start codon for the *metH* gene, which is also changed from GTG to ATG. pKF5.2 and the PCR-generated fragment were digested with *Eco*RI and *Bsp*EI; the resulting PCR-generated fragment and the vector sequence from pKF5.2 were gel purified using the Gene Clean kit, ligated, and transformed into XL1-Blue cells. The

plasmid containing the correct insert was designated pKF3A (Figure 2D).

Site-directed mutagenesis by overlap extension (Horton et al., 1993) was performed to remove the *Hin*dIII site at nt 3147 in the gene by a silent single nucleotide change of G to A. Four primers were used for overlap extension: primer 5 contains nt 2744−2767 of the sense strand, upstream of

5′-TTTTGACTGGCAGGCTTACACGCCG-3′ [primer 5]

5′-CGTTGCGCCGAAaCTTTCTGGTAAA-3′ [primer 6]

5′-TTTACCAGAAAGtTTCGGCGCAACG-3′ [primer 7]

5′-GCTGAAGTACCAACCCGAAACCGAT-3′ [primer 8]

an *Mlu*I site at nt 3051; primer 6 contains nt 3137−3161 of the sense strand with nt 3149 changed from G to A; primer 7 contains sequence complementary to nt 3137−3161 of the sense strand, with nt 3149 changed from C to T; and primer 8 contains sequence complementary to nt 3524−3548 of the sense strand, downstream of the *Acc*I site at nt 3328. Mutagenesis was performed by amplifying the pKF3A template using primers 5 and 7, and in another tube using primers 6 and 8. The products of these reactions were gel purified and used as templates for a third reaction using primers 5 and 8. The fragment generated in the latter reaction was digested with *Acc*I and *Mlu*I, ligated into plasmid pKF3A that had been digested with the same enzymes, and used to transform strain XL1-Blue. A strain containing a plasmid shown to lack the *Hin*dIII site at nt 3147 was termed XL1-Blue/pKF4A (Figure 2E). Dideoxy chain termination sequencing using a Sequenase kit, Version 2.0 (United States Biochemical), was performed to confirm the presence of the desired mutation and to show that no additional mutations were generated during the polymerase chain reaction. The modified *metH* gene was then moved into the expression vector pKK223-3. pKF4A and pKK223-3 were digested with *Eco*RI and *Hin*dIII. The fragment containing the *metH* gene was gene-cleaned, ligated into pKK223-3, and used to transform strain XL1-Blue. The resulting plasmid was designated pKF5A (Figure 2F).

Synthesis of the Gene Module. The overlapping DNA oligomers shown in Figure 3 were synthesized and purified as described in the section on DNA oligomer synthesis. As diagrammed in Figure 4, we were able to use overlap extension and oligomers 1−16 with primers P1 and P7, and

5′-GGTTAGCCATGCCCGAATTC-3′ [primer P1]

5′-CGGTTTCTTGACGCCCGTGCT-3′ [primer P7]

with oligomers 15−22 and primers P2 and P8. The amplification was carried out in a 100 μL solution containing

5′-GGCAGCAGCATTAGCAAGCT-3′ [primer P2]

5′-GCAGAACTACAGCGGACCGACG-3′ [primer P8]

100 mM Tris, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM each of the four nucleotide triphosphates, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), 1 μM each of the two primers, and 0.02 nM each of the oligomers. The reaction mixture lacking the polymerase was incubated at 90 °C for 5 min prior to polymerase addition and 30 cycles of 94 °C (1 min), 55 °C (1 min), and 72 °C (2 min) were carried out using the Programmable Thermal Controller (MJ Research). To amplify the complete module sequence (1376 bp), we used the amplified products from the two reactions described above as templates and P1 and P2 as primers. The products were run on a 1.3% agarose gel, and the 1376 nt band was excised. The DNA was extracted and purified using a Gene Clean II kit (Bio 101). The full length gene fragment was cloned into a TA cloning vector (Invitrogen) and used to transform OneShot competent cells (Invitrogen), supplied with the TA cloning kit. Fifteen of the clones were grown in LB medium, and the plasmid DNA was isolated using the Qiagen miniprep (Qiagen) procedure. The synthetic gene module was isolated from each of these plasmids by digesting with *Eco*RI and *Hin*dIII, subcloned in the *Hin*dIII/*Eco*RI digested pUC19 vector (Life Technologies), and used to transform electrocompetent cells supplied by the manufacturer. DNA preparations from six of these clones were subjected to automated sequencing using a PRISM sequenase terminator double-stranded DNA sequencing kit (Applied Biosystems).

Purification of Methionine Synthase. Cells of strain XL1-Blue containing pMMA-07 were grown aerobically at 37 °C in M9 medium (Miller, 1972) supplemented with 0.4% (w/v) glucose, 100 μg/mL ampicillin, 5 μM hydroxocobalamin, micronutrients (Neidhardt et al., 1974), 10 μM thiamine, and 20 amino acids at the levels previously employed by Wanner et al. (1977). The cells from 6 L of culture were harvested when stationary phase was reached (OD at 420 nm ∼4.5) by centrifugation at 12000*g* for 10 min, washed with 10 mM potassium phosphate buffer, pH 7.2, and stored at −80 °C prior to protein purification. The purification procedure was a modification of the procedure previously described (Banerjee et al., 1989). After disruption of the cells by sonication and ultracentrifugation to remove cell debris, the enzyme was purified by chromatography at 4 °C on DEAE-Sepharose using a 10 × 2 cm column equilibrated with 10 mM potassium phosphate buffer, pH 7.2. The enzyme was eluted with a 400 mL linear gradient from 10 to 500 mM potassium phosphate buffer. The eluted enzyme was concentrated in a pressure cell (Amicon) with a PM30 membrane (Amicon) to ∼20 mL, dialyzed against 50 mM potassium phosphate buffer, and applied to a MonoQ 16/10 FPLC column (Pharmacia) equilibrated at 20 °C with the same buffer. The column was washed with 100 mL of 50 mM potassium phosphate buffer, then with 120 mL of 118 mM potassium phosphate buffer, and finally with a 150 mL linear gradient between 118 and 320 mM potassium phosphate buffer. Methionine synthase elutes at ∼200 mM buffer concentration. The reddish fractions were pooled and concentrated to ∼5 mL, and then exchanged into 10 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA for storage at −80 °C. At this point the enzyme was 90−95% pure as assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. If homogeneous enzyme was required, chromatography on Bio-Gel HTP was performed as previously described (Frasca et al., 1988).

Expression and Preparation of Mutant Forms of Methionine Synthase. Recombinant wild-type methionine synthase from *E. coli* K-12 strain DH5αF′/p4B6.3 was overproduced

as described previously (Banerjee et al., 1989), except that M9 minimal medium was supplemented with 19 amino acids (methionine is omitted because this strain contains *metH* under its own promoter and MetH expression is repressed in the presence of methionine). Mutant methionine synthase proteins were overproduced in *E. coli* K-12 strain XL1-Blue containing plasmid pMMA-07. Mutant strains were constructed by incorporating mutant cassettes into plasmid pM1−38, which contains only the synthetic gene module corresponding to the 27 kDa domain, since the full length *metH* gene lacks the necessary unique restriction sites. Oligonucleotides corresponding to both strands of the desired cassette were synthesized and annealed to generate double-stranded cassettes. In the cassette corresponding to nt 2212−2352, base changes of CAC to GGC and GAC to GAA and AAC generated the mutations His759Gly, Asp757Glu, and Asp757Asn, respectively. In the cassette corresponding to nt 2358−2445 of the *metH* coding region, base changes of TCT to GCT generated the mutation Ser810Ala. In the cassette corresponding to nt 2113 to 2165, base changes of TTT to GCT, TTC to GCC, and CTG to GCG generated the mutations Phe708Ala, Phe714Ala, and Leu715Ala, respectively. Plasmid pM1-38 was cut with *Nhe*I and *Sal*I and ligated with the cassettes containing the His759Gly, Asp757Glu, and Asp757Asn mutations. Plasmid pM1-38 was cut with *Pst*I and *Hpa*I and ligated with the cassette containing the Ser810Ala mutation. Following digestion of plasmid pM1-38 with *Bst*EII and *Xma*1, the cassettes containing the Phe708Ala, Phe714Ala, and Leu715Ala mutations were introduced by ligation. The plasmids were then cut with *Dra*III and *Spl*I, excising the module corresponding to the 27 kDa domain, which was then ligated with *Dra*III−*Spl*I digested pMMA-07, generating the plasmids pMMA-17 (His759Gly), pMMA-21 (Asp757Glu), pMMA-20 (Asp757Asn), pMMA-19 (Ser810Ala), pMMA-14 (Phe708Ala), pMMA-15 (Phe714Ala), and pMMA-16 (Leu715Ala). The mutations were confirmed by automated sequencing of 100 bp on each side of the altered bases. Overexpression and purification of each mutant protein was performed as described for wild-type enzyme.

Enzyme Assay. Methionine synthase was assayed by a recently described nonradioactive assay (Drummond et al., 1995) which measures the conversion of CH_3-H_4folate to H_4folate by derivatization of the latter to CH^+═H_4folate. Briefly, enzyme was activated by incubating for 5 min at 37 °C in 800 μl of 100 mM potassium phosphate buffer, pH 7.2, containing 250 μM (6-*R*,*S*)-CH_3-H_4folate, 19 μM AdoMet, 50 μM hydroxocobalamin, and 25 mM dithiothreitol. Turnover was initiated by the addition of homocysteine (500 μM final concentration). After 10 min at 37 °C, the reaction was quenched with 200 μL of formic acid/hydrochloric acid, and the mixture was incubated at 80 °C to convert H_4folate to CH^+═H_4folate. The derivatized product was quantitated by measuring its absorbance at 350 nm (ϵ_{350} = 26 000 M^{-1} cm^{-1}). One unit of enzyme activity is defined as one micromole of product formed per minute.

RESULTS

Expression of Cobalamin-Dependent Methionine Synthase Holoenzyme from Plasmid pKF5A in E. coli Strain XL1-Blue. Overexpression of methionine synthase as holoenzyme requires a balancing act in which the rate of enzyme synthesis must be coordinated with the rate of cobalamin transport and insertion. When strain DH5αF′ transformed with the original p4B6.3 vector containing the *metH* gene under its own promoter was grown in glucose minimal medium supplemented with hydroxocobalamin, the harvested cells were orange-pink in color and the specific activity measured in the crude extract was ∼0.5 units per mg of protein. In contrast, growth of strain XL1-Blue containing pKF5A in LB medium (Miller, 1972) yielded cells that lacked the orange-pink color and had a specific activity in the crude extract of ∼0.1 units per mg of protein. These studies suggested that, in LB medium, the rate protein synthesis is faster than that of cobalamin transport, so that most of the enzyme is expressed as apoenzyme and degraded before the cells are harvested. To ensure the production of holoenzyme, the rate of enzyme synthesis must not be faster than the rate of cobalamin transport. This can be accomplished by using a strain that expresses the protein continuously during steady-state growth. The vector pKK223-3, in which expression is controlled by the *tac* promoter, allows a moderate level of protein expression in the absence of IPTG and increased protein expression in the presence of IPTG. We chose to use this vector for overexpression of the synthetic *metH* gene.

The plasmid pKF5A contains the *met*H gene incorporated into the expression vector pKK223-3, and we attempted to optimize growth and induction conditions for this construct to obtain maximally active enzyme. We were unable to grow strain XL1-Blue containing plasmid pKF5A in M9 glucose minimal medium, although the same strain lacking pKF5A grows on this medium. Supplementation of M9 glucose medium with amino acids permits growth of the overproducing strain (doubling time ≈65 min). Growth in medium supplemented with micronutrients, hydroxocobalamin, and 20 amino acids produced extracts of much higher specific activity (0.8 units per mg of protein) than growth in LB medium, and much more MetH protein was seen in sonicates of cells grown in the defined supplemented medium (Figure 6, compare lanes 2 and 6 or 3 and 7). Although the level of expression of MetH was much higher when cells were grown in medium containing 50 μM IPTG (Figure 6, compare lanes 6 and 8 or 7 and 9), the specific activity of the enzyme was not increased, suggesting that cobalamin transport was not sufficiently rapid to form holoenzyme in the presence of IPTG. Additional evidence indicating production of apoenzyme when cells were grown in medium containing IPTG was obtained by preincubating the crude extracts with methylcobalamin prior to assay. Activity was increased in crude extracts from cells grown in the presence of IPTG, but not from those grown in the absence of IPTG, suggesting that apoenzyme is present in the induced cells but not in those that are uninduced. The experiments described above allow us to define optimized overexpression conditions for methionine synthase in *E. coli*: continuous overexpression during steady-state growth of XL1-Blue/pKF5A cells in glucose M9 medium supplemented with 20 amino acids and hydroxocobalamin and lacking IPTG.

Gene Module Design and Synthesis. We wished to introduce unique restriction sites into the synthetic gene without altering the amino acid sequence of this region of the protein. We decided not to optimize codon usage throughout the synthetic module but only to change codons as required for introduction of unique restriction sites. This decision was made out of a concern that changes in codon usage might disrupt the timing of folding vs cofactor

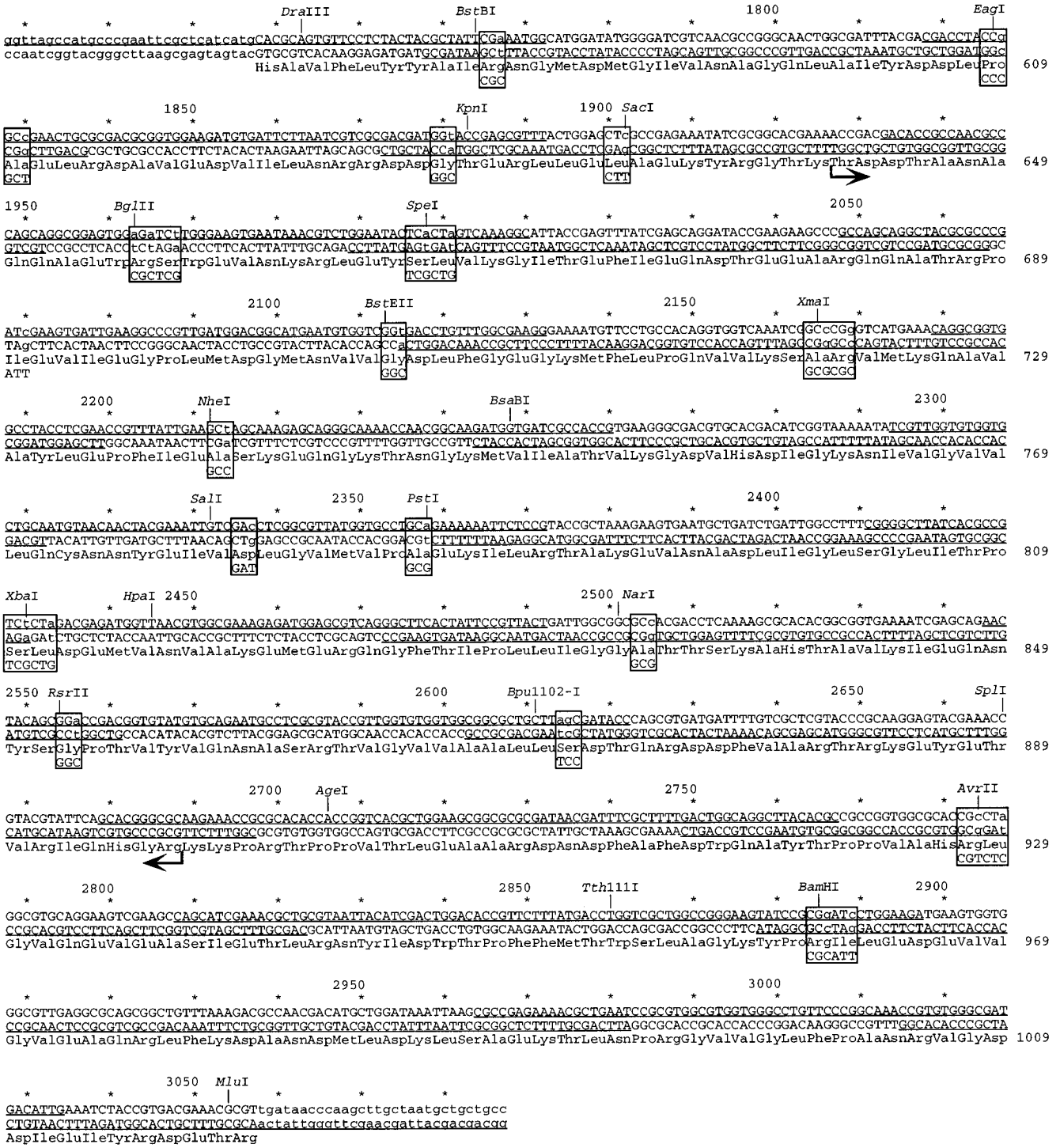

FIGURE 3: DNA sequence of the synthetic cassette is compared with the wild-type sequence. The 27 kDa cobalamin-binding region generated by tryptic digestion extends from amino acid residue 643 to 896 (nt 1927−2688) and is indicated by arrows below the amino acid sequence (Drummond et al., 1993b). The top two lines show the double-strand sequence of the synthetic gene module we constructed. The third line shows deduced amino acid sequence. The codon changes that were made to introduce unique restriction sites are boxed with the changed nucleotides shown in lowercase. Within these boxes, the fourth line shows the original wild-type codons. The first nucleotide in the ORF of methionine synthase was assigned +1 in the gene sequence. The 22 synthetic overlapping DNA oligomers that were synthesized are underlined. The first 30 and last 30 base pairs (lowercase) are random sequences added to introduce *Eco*RI and *Hind*III sites at the beginning and at the end of the synthetic gene cassette, respectively. Preexisting unique restriction sites in this region are also shown in this figure: *Dra*III (nt 1743), *Bsa*BI (nt 2248), *Hpa*I (nt 2445), *Spl*I (nt 2667), *Age*I (nt 2706), *Tth*111I (nt 2860), and *Mlu*I (nt 3054).

insertion. Further, the synthetic module represents only ~25% of the complete *metH* sequence, and optimization of this portion is unlikely to have a dramatic impact on the overall rate of protein synthesis.

The *metH* coding sequence between nt 1741 (a *Dra*III site) and 3053 (an *Mlu*I site) was first translated into an amino acid sequence using the MacVector program (IBI). This amino acid sequence was then reverse-translated to a degenerate nucleotide sequence. We used MacVector to generate a list of restriction enzymes that had sites in the degenerate sequence, eliminating from this list enzymes that already had sites in the wild-type sequence. The nondegenerate sequence was changed to introduce unique restriction sites at ~70−90 bp intervals throughout the region, avoiding

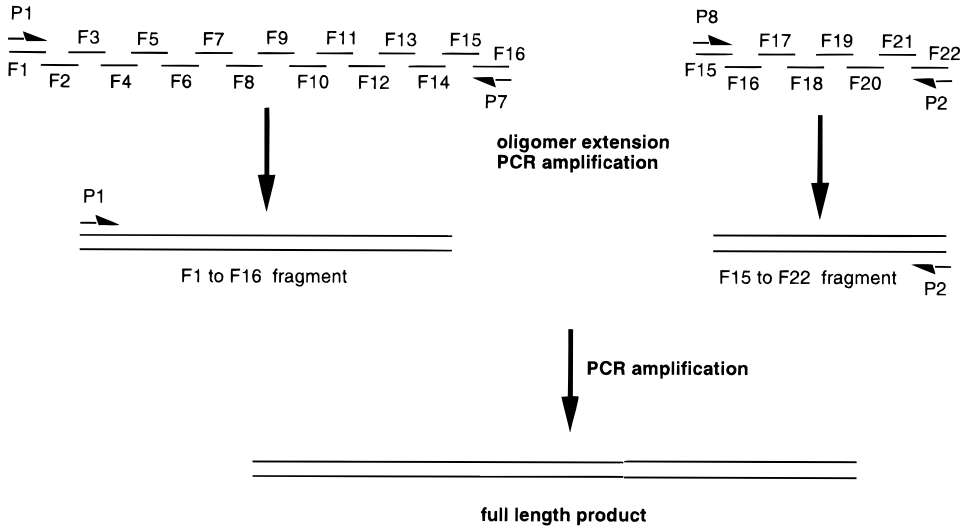

FIGURE 4: Scheme for the overlap extension of oligomers to obtain the synthetic gene module. The overlapping DNA oligomers F1 to F16 (the first 16 underlined sequences in Figure 3) and primers P1 and P7 were used to obtain the F1 to F16 fragment using overlap extension and PCR amplification. Similarly, DNA oligomers F15 to F22 and primers P2 and P8 were used to obtain the F15 to F22 fragment. The products from the two reactions above were used to amplify the full length product using the polymerase chain reaction with P1 and P2 as the primer sequences.

the introduction of the rare codons CGA, CGG, AGG, CTA, TCG, and ATA. The sequence of the synthetic module is compared with the original sequence in Figure 3, and altered codons are boxed with the changed nucleotide(s) shown in lowercase.

The 22 overlapping DNA oligonucleotides indicated by underlining in Figure 3 were synthesized and purified as described in the section on DNA oligomer synthesis. These oligomers varied in length from 70 to 105 nt, and the overlaps between oligomers were from 18 to 20 bp. Attempts to obtain the DNA sequence for the full length gene module by overlap extension and subsequent PCR amplification of the 22 oligomers with appropriate primers were not successful. However, as diagrammed in Figure 4, we were able to use overlap extension and oligomers 1−16 with primers P1 and P7 and with oligomers 15−22 and primers P2 and P8. To amplify the complete module sequence (1376 bp), we used the amplified products from the two reactions described above as templates and P1 and P2 as primers. The full length product was cloned into a TA cloning vector (Invitrogen) and used to transform OneShot competent cells (Invitrogen). DNA preparations from six of these clones were subjected to automated sequencing using a PRISM sequenase terminator double-stranded DNA sequencing kit (Applied Biosystems).

The gene fragment products we obtained after overlap extension and amplification had an average of one error per 150 bp. All clones had more than one error in the sequence, but fortunately at least one clone had the correct sequence in each of the 22 cassettes flanked by unique restriction sites. We observed single and multiple base deletions as well as all possible single base mutations. In the multiple base deletions, the number of bases deleted varied from three to fifteen. Most of the single mutations were either C to T, G to A, or A to G. There were very few purine to pyrimidine or pyrimidine to purine mutations. The scarcity of dT nucleotide mutations may suggest that some of these mutations were due to base changes during deprotection of the long oligomers; the dT phosphoramidite does not have any protecting groups to deblock after the oligomer synthesis. The generation of multiple base deletions is more difficult to understand but may be due to secondary structure formation in the oligomers. Some of the errors are likely due to the fidelity of the *Taq* polymerase that we used, and might be minimized if other, more accurate, polymerases were employed. It is also possible that errors would have been reduced if more stringent methods of oligomer purification had been employed prior to the overlap extension and amplification reactions. To correct the errors in the synthetic module, we started with the DNA sequence with the smallest number of errors and replaced incorrect regions by subcloning correct fragments from the other DNA sequences.

DNA sequencing between the *Dra*III and *Spl*I restriction enzyme sites (Figure 3) of the plasmid pM1-38 that carries the synthetic module was performed to confirm the DNA sequence. *Dra*III and *Spl*I are unique restriction sites in the wild-type methionine synthase gene and allowed introduction of the synthetic module into the wild-type *metH* gene. The *Dra*III−*Spl*I fragment from pM1-38 was subcloned into pKF5A, forming the pMMA-07 plasmid that carries the complete methionine synthase DNA sequence, where the *Dra*III−*Spl*I sequence is replaced by the synthetic module with unique restriction sites at ∼80 bp intervals (Figure 5).

A diagram of the placement of unique restriction sites in the synthetic module extending between the *Dra*III (1743) and *Spl*I (nt 2667) restriction enzyme sites in the semisynthetic *metH* gene is shown in Figure 5. This region encompasses all but the last seven amino acids of the sequence coding for the cobalamin-binding region generated by tryptic digestion, which extends from bp 1927 to 2688. Although a longer synthetic linear sequence was actually assembled, extending to the *Mlu*I site at bp 3060, this portion of the synthetic module codes for the C-terminal activation region of methionine synthase and is being used for the construction of a module extending from the *Spl*I site to the end of the *metH* gene.

Expression of the Semisynthetic Methionine Synthase. When strain XL1-Blue containing plasmid pMMA-07, which contains the semisynthetic *metH* gene in a pKK223-3 expression vector, was grown to stationary phase in glucose

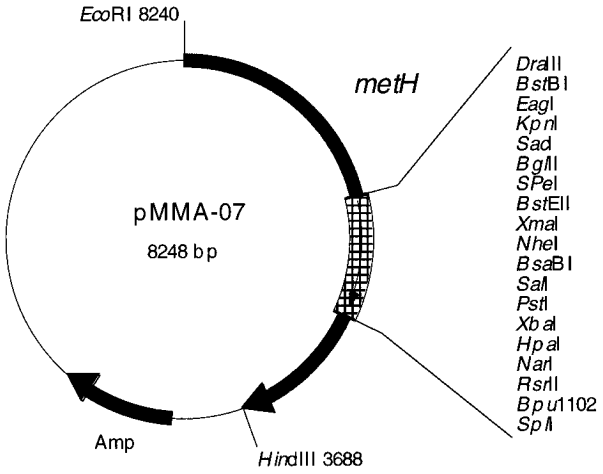

FIGURE 5: Restriction map of plasmid pMMA-07 which overproduces methionine synthase in *E. coli*. The *metH* coding sequence extends from +1 to 3681 and is indicated by a filled arrow. The synthetic gene module is between *Dra*III and *Spl*I sites and is indicated by the checkered area within the *metH* gene. All the engineered and natural unique restriction sites in the synthetic gene module are shown.

M9 minimal medium supplemented with hydroxocobalamin and amino acids, an equivalent level of expression was observed as in strain XL1-Blue containing plasmid pKF5A (Figure 7), which contains the wild-type *metH* gene. Furthermore, similar specific activities were observed in sonicates derived from strains containing the wild-type and semisynthetic genes (0.80 ± 0.05 units per mg of protein). The specific activity of methionine synthase purified from strain XL1-Blue/pMMA-07 was identical within experimental error (7.5 ± 0.8 units per mg) with the specific activity (6.6−7.8 units per mg) of recombinant methionine synthase isolated from DH5aF′/p4B6.3 (Banerjee et al., 1989). Thus the enzyme activity and level of expression do not appear to have been altered by the sequence changes introduced to construct the semisynthetic gene.

Selection of Mutations and Mutation Sites. The recent crystal structure of the 27 kDa cobalamin-binding region pointed to several residues that were likely to be important in modulating the reactivity of the cobalt in methionine synthase (Drennan et al., 1994a,b). These residues include His759, which is coordinated at the lower axial position of the cobalt in methylcobalamin enzyme, and Asp757 and Ser810, which form a hydrogen-bonded network from His759 to solvent water (Figure 1). In methionine synthase, the methylation and demethylation reactions of catalysis involve heterolytic cleavage of the carbon−cobalt bond (Banerjee et al., 1990). The enzyme must alternately stabilize methylcobalamin and cob(I)alamin forms of the prosthetic group (eqs 1 and 2). In model cobalamins, these

$$\text{E·CH}_3\text{-cob(III)alamin} + \text{Hcy} \rightarrow \text{E·cob(I)alamin} + \text{Met} \quad (1)$$

$$\text{E·cob(I)alamin} + \text{CH}_3\text{-H}_4\text{folate} \rightarrow \text{E·CH}_3\text{-cob(III)alamin} + \text{Hcy} \quad (2)$$

forms are six and four coordinate, respectively.

Formation of cob(I)alamin in methionine synthase is thus assumed to involve dissociation of the His759 ligand. Protonation and deprotonation of the His759−Asp757 pair, perhaps mediated by Ser810, may play an important role in interconversion between methylcobalamin and cob(I)alamin species (Drennan et al., 1994a,b). The His-Asp-Ser ligand triad is conserved in all the five known sequences of methionine synthase: *E. coli* (Banerjee et al., 1989; Drummond et al., 1993b), *Mycobacterium leprae* (Smith, 1994), *Haemophilus influenzae* (Fleischmann et al., 1995), *Caenorhabditis elegans* (Swinburne, 1994), and *Synechocystis sp.* (Kaneko et al., 1995). For this initial study we chose to mutate His759 to glycine, eliminating the lower ligand to the cobalt. The role of a His-Asp pair in modulating the reactivity of the heme iron in cytochrome c peroxidase has recently been examined (Goodin & McRee, 1993), and we followed the lead of these authors by making the relatively conservative mutations Asp757 to asparagine and Asp757 to glutamate. These mutations were expected to allow His759 to coordinate the cobalt but to perturb the hydrogen bonding and the electrostatic interations that affect protonation/deprotonation. Replacement of Ser810 by alanine constituted a first test of the importance of a hydroxyamino acid at the external solvent-exposed site of the hydrogen-bonded network.

Three residues in the α-helical domain that caps the top face of the corrin ring of cobalamin in methionine synthase were also chosen for mutagenesis. Since the methyl tranfer reactions occur at the top face of the corrin, the hydrophobic cap residues may play some role in the reaction cycle, most likely by affecting substrate binding or product release. As shown in Figure 1, replacement of Phe708 or of Leu715 with alanine is expected to increase significantly the solvent accessibility of the methyl group of methylcobalamin, while replacement of Phe714 with an alanine should have a much less dramatic effect.

Steady-State Activities of the Mutant Proteins. The V_{max} and the K_M values for CH_3-H_4folate and homocysteine were determined for each of the seven mutants and are reported in Table 1. All of the mutant proteins except His759Gly display significant activity in steady-state assays. The His759Gly enzyme has an apparent activity <0.5% of wild-type enzyme. Since the mutant proteins were expressed in an *E. coli* strain that has a viable chromosomal *metH* gene, a small amount of wild-type enzyme contaminates the mutant protein preparations. (Several attempts to produce a *metH* knockout strain in our laboratory have failed.) The activities reported in Table 1 should therefore be considered upper limits. In fact, enzyme-monitored stopped-flow turnover experiments described in the accompanying paper (Jarrett et al., 1996) suggest that the His759Gly mutant is completely inactive in overall catalysis. The V_{max} and K_M values for the other proteins with mutations in the ligand triad (Asp757Asn, Asp757Glu, and Ser810Ala) point to the relative importance of each of these residues in catalysis. The Ser810Ala enzyme is ∼50% active, while the Asp757 mutant proteins are ∼4−6% active, suggesting that while each of these residues contributes significantly to catalysis, they are not essential. Mutations in the ligand triad also affect the K_M values for CH_3-H_4folate and homocysteine. Mutations of cap residues Phe708 and Leu715 affect V_{max} (6% and 9% of wild-type activity, respectively), and the Phe708Ala and Leu715Ala mutations lead to increased K_M values for homocysteine and CH_3-H_4folate, respectively. In the mechanism deduced for methionine synthase [(Banerjee et al., 1989; see also Figure 2 of the accompanying paper (Jarrett et al., 1996)], K_M values depend on combinations of

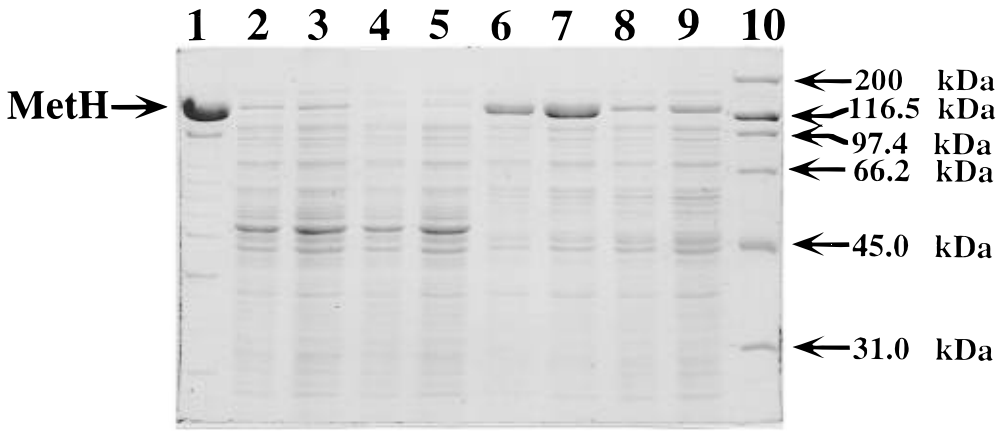

FIGURE 6: Electrophoretic analysis of crude cell extracts of strain XL1-Blue/pKF5A under denaturing conditions. Lane 1, purified MetH; lanes 2 and 3, 10 and 20 μg of soluble sonicate protein from cells grown in LB in the presence of IPTG; lanes 4 and 5, 10 and 20 μg of soluble sonicate protein from cells grown in LB in the absence of IPTG; lanes 6 and 7, 10 and 20 μg of soluble sonicate protein from cells grown in glucose MOPS medium supplemented with 20 amino acids, thiamine, cobalamin, trace metals (Neidhardt et al., 1974), and IPTG; lanes 8 and 9, cells grown in the defined supplemented glucose MOPS medium without IPTG; lane 10, molecular weight standards.

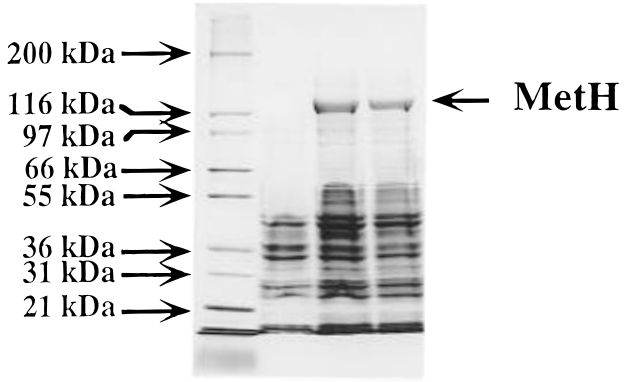

FIGURE 7: Gradient SDS−polyacrylamide gel of soluble fractions of *E. coli* cell extracts that overproduce wild-type and semisynthetic methionine synthase. The gradient is from 8−16% polyacrylamide. Lane 1, protein size markers; lanes 2−4, soluble fractions of sonicates of XL1-Blue *E. coli* cells with plasmids pKK233-3 (control), pKF5A (wild-type methionine synthase) and pMMA-07 (semisynthetic methionine synthase), respectively. Comparison of the *metH* expression in lanes 3 and 4 indicates that the overexpression of methionine synthase is not affected by changes in the gene sequence.

several rate constants and are not equivalent to binding constants. Indeed, mutations in the B_{12}-binding region are not expected to affect substrate binding directly. We have shown that both CH_3-H_4folate and homocysteine are bound to the portion of methionine synthase that lies upstream of the cobalamin-binding region (Goulding and Matthews, unpublished data).

The results reported in Table 1 were obtained with an assay that makes use of a chemical reducing system (dithiothreitol and hydroxocobalamin) for reactivation of cob(II)alamin enzyme. To ensure that these results are not specific to this particular choice of assay method, we performed two control experiments. We first assayed each ligand triad mutant under anaerobic conditions with the physiological reducing system: flavodoxin, ferredoxin (flavodoxin)-$NADP^+$ oxidoreductase, and NADPH. The relative activities, measured using the physiological reducing system, were similar to those obtained using the dithiothreitol/hydroxocobalamin reducing system. The activity assay we routinely use measures the formation of H_4folate from CH_3-H_4folate (Drummond et al., 1995) but not the methylation of homocysteine. To eliminate the possibility that some mutant proteins were not coupling formation of H_4folate to the methylation of homocysteine, we next separately measured H_4folate formation from CH_3-H_4folate and [^{14}C-*methyl*]methionine formation from homocysteine and [^{14}C-*methyl*]CH_3-H_4folate (Banerjee et al., 1989) for the Ser810, Asp757, and F708 mutant enzymes. Each each of these mutant enzymes catalyzes formation of both products at the same rate, demonstrating that the activities observed are due to complete transfer of a methyl group from CH_3-H_4folate to homocysteine.

Properties of the Cobalamin Cofactor in the Mutant Proteins. Changes in the properties of the cofactor in the mutant enzymes were evident from the visible spectra of the mutant proteins as isolated (Table 1). The wild-type methionine synthase was purified from overproducing strain XL1-Blue/pMMA-07 with the prosthetic group present as methylcobalamin. In contrast, although all mutant enzymes contained bound cobalamin, six of the seven mutant proteins were purified with bound cob(II)alamin or cob(III)alamin, as shown in Table 1. Isolation of the prosthetic group in the cob(II)alamin or cob(III)alamin form rather than the methylcobalamin form suggests that the methylated mutant enzymes are less stable toward photolysis than the wild-type enzyme during isolation of the protein without protection from ambient light. Indeed, mutations of His759, Asp757, and Ser810 lead to increased rates of photolysis of the methylcobalamin prosthetic group (Jarrett et al., 1996). Mutations of Phe708 and Leu715 to Ala also lead to dramatic increases in the rate of photolysis (Jarrett et al., manuscript in preparation). In contrast, the side chain of Phe714 does not form part of the hydrophobic cage surrounding the upper face of methylcobalamin (Figure 1), and the Phe714Ala mutant is isolated in the methylcobalamin form.

When wild-type enzyme is demethylated by photolysis or anaerobic treatment with homocysteine, the enzyme-bound cob(II)alamin prosthetic group is resistant to further oxidation. The stability of the enzyme-bound cob(II)alamin in aerobic solution stands in marked contrast to the properties of free cob(II)alamin, which undergoes rapid oxidation to aquocob(III)alamin. Aquocob(III)alamin has a water molecule coordinated to the upper axial position of the cobalt. The Asp757Glu and Asp757Asn mutant proteins are readily oxidized to cob(III)alamin, and the Ser810Ala mutation slowly oxidizes to cob(III)alamin on storage at 4 °C.

Table 1. Steady State Parameters for Methionine Synthase Mutants[a]

mutant enzyme	K_M Hcy (μM)	K_M (6*S*)-CH_3-H_4folate (μM)	V_{max}/E_T (37 °C) (s^{-1})	enzyme form isolated[b]
wild-type	15 (1)	35 (1)	27.7 (1)	CH_3-cobalamin
Ser810Ala	39 (2.6)	80 (2.3)	15.4 (0.56)	cob(II)alamin
Asp757Asn	12 (0.8)	26 (0.74)	1.74 (0.063)	cob(II/III)alamin
Asp757Glu	36 (2.4)	8.8 (0.25)	0.97 (0.035)	cob(III)alamin
His759Gly	ND[c]	ND[c]	<0.13 (<0.005)	cob(II)alamin
Phe708Ala	104 (6.9)	36 (1.0)	1.56 (0.056)	cob(II)alamin
Phe714Ala	45 (3.0)	50 (1.4)	20.0 (0.72)	CH_3-cobalamin
Leu715Ala	15 (1.0)	117 (3.3)	2.56 (0.092)	cob(II)alamin

[a] Steady-state parameters were determined at 37 °C using the spectrophotometric assay described in Materials and Methods. The numbers in parentheses represent fold increases or decreases as compared to wild-type enzyme. [b] This column describes the predominant form of the cofactor in each mutant protein as isolated by the enzyme preparation method described in Materials and Methods. [c] Not determined.

Cobalamin in these ligand triad and cap mutations behaves more like the free cofactor, which is readily oxidized in the cob(II)alamin state and labile toward photolysis in the methylcobalamin state. These findings indicate the important role of residues of the ligand triad and cap in modifying the properties of the bound cofactor.

DISCUSSION

The overexpression of enzymes that contain cofactors poses potential problems, particularly if the cofactor is normally inserted before the nascent peptide leaves the ribosome and is required for proper folding of the protein. These problems may be particularly severe if the cofactor is not synthesized in the cell but must be transported from the medium. In this paper, we describe growth conditions that enable efficient overexpression of methionine synthase holoenzyme and that may be applicable to the overexpression of other cobalamin-dependent enzymes.

Having optimized overexpression of the wild-type protein, we set out to generate a series of mutations in the cobalamin binding domains of methionine synthase. Among the several methods that are available for site-directed mutagensis (Kunkel, 1985; Sugimoto, 1989), cassette mutagenesis is the most convenient. This method is especially preferable when the gene involved is too long to be completely sequenced with one set of primers. The main limitation of this method is the availability of closely spaced unique restriction sites in the gene. The region between *Dra*III and *Spl*I in the *metH* gene, which encompasses all but seven residues of the cobalamin-binding region, initially contained only two unique restriction sites. The construction of a semisynthetic gene for methionine synthase in which unique restriction sites have been introduced at ~80 bp intervals in the sequence coding for the cobalamin-binding domains permits facile cassette mutagenesis (Reidhaar-Olson et al., 1991). Chemical synthesis of a fragment coding for this region employed a PCR-mediated gene synthesis strategy (Holler et al., 1993) and led to the introduction of 15 additional unique restriction sites, spaced at 15−115 nt intervals in the semisynthetic sequence. The enzyme sites we selected are cleavage sites for commercially available enzymes, and we avoided adjacent sites that would produce self-complementary cohesive ends on the individual cassettes. Engels and Uhlmann (1989) have described several methods for gene synthesis. Synthetic genes can be used to introduce restriction enzyme sites without changing the amino acid sequence (Climie & Santi, 1990), to optimize codon usage for protein expression in a heterologous host (Hernan et al., 1992), or to express *de novo*-designed peptides and proteins.

One of the uses of synthetic genes is to optimize the codon usage of the gene by replacing rare codons with more frequently used codons in the organism used for expression. In this work we decided against this strategy, due to the large size of the gene and the possibility that translational pausing might be important for proper folding of the individual domains. Using the TRP3 gene, it has been shown that translational pausing promotes the correct *in vivo* folding of protein (Crombie et al., 1992). We were concerned that removing rare codons from the wild-type gene sequence might interfere with binding of the cobalamin prosthetic group during folding. For the same reason, we also tried to avoid silent mutations that would introduce the rare codons CGA, CGG, AGG, CTA, TCG, and ATA into our synthetic module, as these might introduce pauses in the wrong place in the sequence.

The synthetic module has been used to construct seven mutations by cassette mutagenesis. Mutations of residues that contact the upper face of the corrin ring (Phe708Ala and Leu715Ala) lead to changes in the rate of steady-state turnover, suggesting that these residues play an auxilliary role in catalysis. These residues may make important nonbonded contacts which are involved in moving the cap domain to allow substrate access to the methylcobalamin prosthetic group. These residues also dramatically affect the stability of the prosthetic group toward light, suggesting that another important role for the N-terminal helical domain of the cobalamin binding region may be to create a hydrophobic protein cage that shields the prosthetic group from adventitious reactions, as initially proposed (Drennan et al., 1994a). The His759Gly mutation, which removes the lower axial ligand to the cobalt in enzyme-bound cobalamin, has crippling effects on the methyl transfer reactions of the catalytic cycle. This choice of mutations is rather drastic, and, in addition to eliminating the ligand to the cobalt, it may have affected the hydrogen bonding of Asp757 to other residues (see Figure 1). More conservative mutations such as histidine to glutamine have been shown to retain activity in cytochrome c peroxidase (Sundaramoorthy et al., 1991); this mutation will be interesting to explore in methionine synthase. Mutations of the Ser810 and Asp757 residues that are linked by a hydrogen bond network to His759 lead to significant decreases in steady-state activity, suggesting that these residues may be important in modulating the properties of the histidine ligand but are not themselves essential for catalysis. The accompanying paper (Jarrett et al., 1996) examines the chemistry and spectral properties of cobalamin

in the enzymes with mutations in the ligand triad and analyzes in more detail the reactivity and stability of the cofactor in these mutant enzymes.

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