The last step in coenzyme B<sub>12</sub> synthesis is localized to the cell membrane in bacteria and archaea

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In *Salmonella enterica*, the last step of the synthesis of adenosylcobamide is catalysed by the cobalamin synthase enzyme encoded by the *cobS* gene of this bacterium. Overexpression of the *S. enterica* *cobS* gene in *Escherichia coli* elicited the accumulation of the phage shock protein PspA, a protein whose expression has been linked to membrane stress. Resolution of inner and outer membranes of *S. enterica* by isopycnic density ultracentrifugation showed CobS activity associated with the inner membrane, a result that was confirmed using antibodies against CobS. Computer analysis of the predicted amino acid sequence of CobS suggested it was an integral membrane protein. Results of experiments performed with strains carrying plasmids encoding CobS–alkaline phosphatase or CobS–β-galactosidase protein fusions were consistent with the membrane localization of the CobS protein. Modifications to the predicted model were made based on data obtained from experiments using protein fusions. The function encoded by the *cobS* orthologue in the methanogenic archaeon *Methanobacterium thermoautotrophicum* strain ΔH compensated for the lack of CobS during cobalamin synthesis in *cobS* strains of *S. enterica*. Cobalamin synthase activity was also detected in a membrane preparation of *M. thermoautotrophicum*. It was concluded that the assembly of the nucleotide loop of adenosylcobamides in archaea and bacteria is a membrane-associated process. Possible reasons for the association of adenosylcobamide biosynthetic enzymes with the cell membrane are discussed.

**INTRODUCTION**

*Salmonella enterica* serovar Typhimurium LT2 synthesizes adenosylcobamide (AdoCba) *de novo* under anaerobic conditions (Jeter et al., 1984). This facultative anaerobic enterobacterium can salvage incomplete corrinoids [e.g. cobyric acid (Cby) and cobinamide (Cbi)] from its environment and convert them into AdoCba under both aerobic and anaerobic conditions (Brushaber et al., 1998; Jeter et al., 1984; Keck et al., 1998). The resulting AdoCba functions as coenzyme for 1,2-propanediol dehydrase and ethanolamine ammonia-lyase enzymes in this bacterium, and is the source of the non-adenosylated cobamide used by methionine synthase (Babior, 1982; Taylor & Weissbach, 1973; Toraya & Fukui, 1982). The current model for the late steps in *de novo* AdoCba biosynthesis and the pathway for salvaging incomplete exogenous corrinoids in *S. enterica* are shown in Fig. 1. Salvaging of Cby requires first its conversion to adenosylcobyric acid (AdoCby) by the ATP:corrinoid adenosyltransferase (CobA) enzyme, followed by its conversion to adenosylcobinamide-phosphate (AdoCbi-P) by the Cbi-P synthase (CbiB) enzyme (Brushaber et al., 1998; Thomas & Escalante-Semerena, 2000). In contrast to Cby, Cbi is directly phosphorylated by CobU, the bifunctional kinase/guanylyltransferase enzyme whose kinase activity is dedicated to Cbi salvaging (Thomas & Escalante-Semerena, 2000). The lower ligand base in the structure shown in Fig. 1 is 5,6-dimethylbenzimidazole; the adenosylated form of 5,6-dimethylbenzimidazolylcobamide is known as adenosylcobalamin (AdoCbl). It should be noted that, during *de novo* synthesis (i.e. under anoxic growth conditions), *S. enterica* incorporates adenine as the lower ligand of the cobamide, yielding a cobamide known as pseudo-B<sub>12</sub> (Keck & Renz, 2000; Stupperich et al., 1987; Trzebiatowski & Escalante-Semerena, 1997). The final step in AdoCba biosynthesis is catalysed by the cobalamin synthase enzyme, CobS (Cameron et al., 1991; Maggio-Hall & Escalante-Semerena, 1999).

Data obtained in this study from isopycnic ultracentrifugation experiments, Western blot analysis, and topological
analysis using alkaline phosphatase and β-galactosidase as reporters are consistent with the conclusion that the CobS enzyme is localized to the cell membrane in bacteria and probably also in archaea. It is shown that in *Escherichia coli*, high levels of this enzyme elicit a membrane stress response detectable by the accumulation of the PspA protein encoded by the *psp* operon.

Functions encoded by the *psp* operon play a role in responding to changes in the energy level of the cell. PspA, an inner-membrane protein, is believed to prevent the loss of protons across the inner membrane, maintaining the proton-motive force (Brissette *et al.*, 1990; Kleerebezem *et al.*, 1996; Model *et al.*, 1997). The implications of a membrane-associated synthesis of coenzyme B$_{12}$ are discussed.

**METHODS**

**Strains and growth conditions.** Strains used in this work and their genotypes are listed in Table 1. *E. coli* strain JE4748 was constructed by moving the kanamycin (Km) resistance marker from strain J134 into strain BL21(DE3) using phage P1 generalized transduction (Davis *et al.*, 1980). *E. coli* strains were routinely grown in Luria–Bertani (LB) rich medium or M9 minimal medium. In rich medium, Km and ampicillin (Ap) were present at 50 mg ml$^{-1}$ and 100 mg ml$^{-1}$, respectively, where applicable. M9 medium was supplemented with Ap (75 mg ml$^{-1}$) for the expression of proteins. Protein expression was induced by the addition of IPTG (0.4 mM final concentration) when cultures reached a cell density of 1 as measured using a Spectronic 20D spectrophotometer set at 650 nm (Milton Roy). *S. enterica* strain TR6583 was grown on no-carbon E (NCE) medium (Berkowitz *et al.*, 1968) supplemented with succinate (30 mM), 1,2-propanediol (10 mM), (CN)$_2$Cbi (15 nM) and trace minerals (Balch & Wolfe, 1976) at 30°C for optimal induction of the *cob* operon (Rondon & Escalante-Semerena, 1992). *S. enterica* strains JE5896 and JE6002 were grown in LB medium to facilitate spheroplast formation (see below). The chromophore 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 mg ml$^{-1}$) or 5-bromo-4-chloro-3-indolyl β-D-galactoside (XGal; 20 mg ml$^{-1}$) was added to the medium as an indicator of alkaline phosphatase (PhoA) or β-galactosidase activity, respectively. For growth curves, strains were grown overnight in LB broth and washed once with sterile saline. Approximately $2 \times 10^6$ c.f.u. were inoculated into 200 ml of fresh medium in 96-well microtitre dishes. Growth was monitored with an ELx808 high-throughput spectrophotometer (Bio-Tek Instruments). OD$_{600}$ readings were taken every 15 min for 18 h. Cultures were shaken for 500 s between measurements. The incubation chamber was maintained at 37°C. Each culture had two replicates.

**Plasmid construction.** All plasmid constructions were verified by DNA sequencing.

**Plasmids pCOBS4 and pNLA1.** The construction of these plasmids was described by Maggio-Hall & Escalante-Semerena (1999).

**Plasmids pCOBS1 and pCOBS6.** Plasmid pCOBS1 was derived from pJO21, a plasmid previously described (O’Toole *et al.*, 1993). The HindIII–HindIII fragment of pJO21 was cloned into cloning...
Table 1. Strains and plasmids

Unless otherwise stated, all strains and plasmids were constructed during the course of this work.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
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<td></td>
</tr>
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<td>BL21(DE3)</td>
<td>F’ ompT hsdSb (rB mB) gal dcm (DE3)</td>
<td>Novagen</td>
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<td>J134</td>
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<td>Weiner et al. (1991)</td>
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<tr>
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<td>This work</td>
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<td>CC118</td>
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<td>Manoil &amp; Beckwith (1985)</td>
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<td></td>
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<tr>
<td>TR6583 (formerly SA2929)</td>
<td>metE205 ara-9</td>
<td>K. Sanderson via J. Roth</td>
</tr>
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<td>lacZYA+ bla+</td>
<td>Casadaban et al. (1983)</td>
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*Abbreviation of Tn10Δ16Δ17 (Way et al., 1984).
†ORF112 of *M. thermoautotrophicum* strain ΔH.

vector pGEM3Zf(−) (Promega) cut with the same restriction enzymes. To construct pCOBS6, the EcoRI–HindIII fragment of pCOBS1 was cloned into pT7-5 cut with the same restriction enzymes.

**Plasmids pCOBS9 and pCOBS10.** Plasmids pCOBS9 and pCOBS10 encode N-terminally truncated products of the cobS gene, directing translation to begin at the second (pCOBS9) or third (pCOBS10) codons for methionyl residues of the gene sequence (Roth et al., 1993). These plasmids were constructed by site-directed mutagenesis of plasmid pCOBS2 (Maggio-Hall & Escalante-Semerena, 1999) to create *NdeI* restriction sites adjacent to each of the new start codons using the reverse primer (5’-AGGGATCCATGACCATG-3’) and 5’-TACGTCAGGCTCAAGTACG-3’ for pCOBS9) or 5’-TACGTCAGGCTCAAGTACG-3’ (for pCOBS10). The new cobS alleles were cloned into plasmid pT7-7, which provides a strong ribosome-binding site (Tabor, 1990). Bases shown in bold-type face identify the engineered *NdeI* site in both primers.

**Plasmid pCOBS16.** The *M. thermoautotrophicum* strain ΔH ORF1112, predicted by sequence homology to encode a cobalamin synthase orthologue, was amplified from chromosomal DNA using PCR protocols. The 5’ primer used created an *NdeI* restriction site immediately upstream of the predicted start codon of ORF1112 to facilitate cloning into pT7-7 (Tabor, 1990). The primers used were: 5’-AGGGATCCATGACCATG-3’ and 5’-CGAGGCG-GAGGCG-GAGGCG-3’. Bases shown in bold-type face identify the engineered *NdeI* site.

**Plasmids encoding CobS–LacZ and CobS–PhoA fusion proteins.** *PstI* restriction sites were engineered into the cobS gene of pCOBS6 for insertion of *phaA* or *lacZ* genes at desired positions using Stratagene’s QuikChange XL Site-Directed Mutagenesis kit according to the manufacturer’s instructions. All plasmids were sequenced to verify the location of the restriction site. Due to the extensive list of plasmids generated, they are not detailed here; specific information on fusion plasmids is available from the authors upon request.

The *phaA*-containing *PstI* fragment from plasmid pCH40 (Hoffman & Wright, 1985) or the *lacZ*-containing *PstI* fragment of plasmid pSKS107 (Casadaban et al., 1983) was cloned into the engineered *PstI* sites of cobS in plasmid pCOBS6. The ligation reactions were then transformed into a *phaA lacZ* null strain (CC118). Since the *phaA* or
lacZ fragment could insert in either orientation, plasmids were screened by restriction analysis to determine the presence and orientation of the phoA or lacZ gene. Properly oriented genes were confirmed by DNA sequencing. The resulting plasmids encoded CobS–PhoA or CobS–LacZ fusion proteins in which the phoA or lacZ gene was preceded by varying lengths of cobS in-frame. A list of the plasmids used in these studies is presented in Table 3.

Electrophoretic analysis. Culture samples were taken immediately prior to induction with IPTG and 2, 4 and 24 h after induction. To correct for differences in the number of cells amongst the samples, the volume of each sample to be taken was determined after measuring the cell density of the culture (Jensen, 1998). Cells were pelleted by centrifugation in a Microfuge 18 (Beckman Coulter) at 21 000 g. Cells were resuspended in 30 ml sample buffer (Fraser et al., 1999), and heated at 95 °C for 10 min. Samples were stored at −20 °C.

Protein purification. A cell-free extract was prepared as previously described from cells of a culture of E. coli BL21(DE3) carrying plasmid pCOBS4 harvested 4 h after induction (O’Tooile & Escalante-Semerena, 1995). Purification of the overproduced protein was monitored by SDS-PAGE (Laemmli, 1970). Unless otherwise noted, all chromatography resins used were equilibrated with 0 mM Tris/HCl buffer, pH 7, at a flow rate of 2 column volumes h$^{-1}$. Proteins that remained bound to the column were eluted with 1 M cyanocobalamin. Proteins that remained bound to the resin after loading, the column was washed with 3 bed volumes of equilibrating buffer followed by buffer containing 1 mM potassium phosphate buffer, pH 7, at a flow rate of 2 column volumes h$^{-1}$.

Step 1. Anion-exchange chromatography was performed on Toyopearl DEAE-650M resin (TosoHaas; 25 × 82 mm, 40 ml bed volume). A total of 11-5 mg protein was loaded per ml resin. The column was developed with a 0-0-3 M KCl linear gradient in buffer at a rate of 2 column volumes h$^{-1}$.

Step 2. Hydroxyapatite chromatography (Bio-Gel HTP Gel, Bio-Rad) was performed on a 25 × 60 mm (30 ml bed volume) column equilibrated with 10 mM potassium phosphate, pH 7. Pooled fractions from step 1 (14-5 mg protein) were applied to dialysis in 4 litres 0-1 M Tris/HCl buffer pH 8 at 4 °C. Proteins adsorbed to the column were eluted with a 10–400 mM linear gradient of potassium phosphate buffer, pH 7, at a flow rate of 2 column volumes h$^{-1}$.

Step 3. Gel filtration chromatography was performed on a 15 × 283 mm (50 ml bed volume) Sephacryl S300 column (Amersham Pharmacia). The void volume of the column was calculated using Blue Dextran (Sigma). Fractions containing overproduced protein from step 2 (4 mg protein) were concentrated and applied to the column at a flow rate of 10 ml h$^{-1}$.

Step 4. A B$_2$$_2$ agarose affinity chromatography column (Sigma; 10 × 30 mm, 2-5 ml bed volume) was used as the last step in the purification protocol since the protein of interest was initially believed to be cobalamin synthase. Fractions containing the overproduced protein from step 3 were pooled and loaded onto the column (1 mg protein). After loading, the column was washed with 3 bed volumes of equilibrating buffer followed by buffer containing 1 mM cyanocobalamin. Proteins that remained bound to the column were desorbed with buffer containing 0-5 M NaCl.

N-terminal sequencing. Fractions from step 3 that contained the overexpressed protein were subjected to SDS-PAGE. The PAGE gel was blotted onto an Immobilon P membrane using a ProBlot apparatus (Bio-Rad) according to the manufacturer’s instructions. The blot was stained with Coomassie brilliant blue R-250 (Sigma) (Sasse, 1991). The protein band corresponding to the 26 kDa protein was excised, dried under a stream of N$_2$, and the N-terminal sequence determined at the Protein Research Laboratory at the University of Illinois-Chicago.

In vitro enzyme activity assays. Succinate dehydrogenase (Sdh) activity assays were performed as described by Markwell & Lascelles (1978). One unit (U) of Sdh activity was defined as the amount of enzyme that synthesizes 1 μmol product min$^{-1}$. Cobalamin synthase (CobS) assays were performed as previously described (Maggio-Hall & Escalante-Semerena, 1999). Cobalamin synthase activity of the Methanobacterium thermautotrophicum enzyme was routinely assayed at 50 °C. One unit (U) of cobalamin synthase activity was defined as the amount of enzyme required to generate 1 μmol of product min$^{-1}$.

β-Galactosidase activity assay. Whole-cell β-galactosidase assays were performed in 96-well microtitre dishes using the fluorogenic substrate 3-carboxyumbelliferyl β-d-galactopyranoside (CUG; Molecular Probes) according to the manufacturer’s instructions. Cultures were grown overnight with aeration at 37 °C in LB medium containing ampicillin. A sample (0-02%, v/v) from the overnight culture was used to inoculate 5 ml of the same medium, grown for 2 h (approximate OD$_{650}$ 0-3) and concentrated threefold into sterile 0-145 M NaCl. Cells were permeabilized by the addition of 50 μl CHCl$_3$ to 300 μl of cells followed by vortexing; 50 μl of permeabilized cells was used per assay. E. coli β-galactosidase (Sigma) was used to generate a standard curve ranging from 1 ng to 10 pg. Reactions were started by the addition of 110 nmol CUG substrate (100 μl 0-1 M sodium phosphate buffer pH 7-3, at 25 °C, containing 1-1 mM CUG, 1 mM MgCl$_2$ and 45 mM β-mercaptoethanol). Reaction mixtures were incubated at 30 °C for 30 min, and reactions were stopped by the addition of 10 μmol Na$_2$CO$_3$ (50 μl 0-2 M Na$_2$CO$_3$). Commercially available 7-hydroxycoumarin-3-carboxylic acid (Molecular Probes), the fluorescent product of the hydrolysis of CUG, was used as standard. Fluorescence was measured using a high-throughput SpectraMax GeminiEM spectrofluorometer (Molecular Devices) with the emission wavelength set at 460 nm and the excitation wavelength set at 390 nm. The amount of cells used per assay was determined by viable cell counts. The number of active molecules of β-galactosidase was calculated taking into consideration that the active form of the enzyme is a tetramer.

Alkaline phosphatase assay. Whole-cell alkaline phosphatase assays were performed in 96-well microtitre dishes using the fluorogenic substrate 4-methylumbelliferyl phosphate (MUP; Molecular Probes). The above procedure for the β-galactosidase assay was modified for the use of MUP and the buffers used were as described by Brickman & Beckwith (1975). Cells were grown under the same conditions as described above and 50 μl of the prepared cells was used per assay. It was not necessary to permeabilize the cells prior to assaying. Shrimp alkaline phosphatase (Promega) was used to generate a standard curve ranging from 1 ng to 10 pg. Reactions were started with the addition of 110 nmol MUP substrate (100 μl 1 mM MUP in 1 M Tris/HCl buffer pH 8-0, at 25 °C). Reaction mixtures were incubated at 30 °C for 30 min and stopped by the addition of 50 μmol K$_2$HPO$_4$ (50 μl 1 M K$_2$HPO$_4$). Commercially available 7-hydroxycoumarin (Molecular Probes), the fluorescent product of the hydrolysis of MUP, was used as standard. Fluorescence was then measured using the high-throughput SpectraMax GeminiEM spectrofluorometer (Molecular Devices) with the excitation wavelength set at 449 nm and the emission wavelength set at 504 nm. The number of cells used per assay was determined by viable cell counts. The amount of active alkaline phosphatase per cell was determined taking into consideration that the active form of the enzyme is a dimer.

Cell fractionation of S. enterica

Procedure 1. Preparation of crude ribosomes. Crude ribosomes were prepared as described by Spedding (1990) from cells of strain TR6583. Briefly, an S30 extract was obtained from cells disrupted using a French pressure cell (Amino) and centrifuged at 30 000 g.
The extract was then centrifuged at 100 000 g using a 70.1 Ti rotor in a Beckman L8-70 refrigerated ultracentrifuge (Beckman Coulter). The resulting pellet containing the ribosomes was brown and translucent. Both pellet and supernatant were assayed for cobalamin synthase activity.

**Procedure 2. Preparation of salt-washed ribosomes.** A more stringent purification of ribosomes was accomplished by centrifuging the S30 extract described above through a cushion containing 1·1 M sucrose and 0·5 M NH₄Cl, as described by Spedding (1990). The resulting pellet was clear. Both pellet and supernatant were assayed for Sdh and CobS activities. This procedure was also repeated without addition of NH₄Cl to the sucrose cushion. The pellet obtained was similar to but smaller than that found in the crude preparation, and both supernatant and pellet were assayed for Sdh and CobS activities.

**Procedure 3. Spheroplast formation and membrane fractionation by isopycnic density ultracentrifugation.** The procedure used to convert *S. enterica* cells to spheroplasts has been described (Osborn & Munson, 1974). Attempts to generate spheroplasts from cells grown on minimal NCE medium were unsuccessful. Although spheroplasts of strain TR6583 grown on LB medium were obtained, these cells did not contain quantifiable levels of CobS activity because of the low expression of the *cob* operon in rich medium (Escalante-Semerena & Roth, 1987). Strain JE5896, which carried *cobS* on a multi-copy number plasmid (pNL1A1), was used instead. It was found that low but detectable levels [0·01 U (mg protein)]⁻¹ of CobS were expressed from plasmid pNL1A1 in strains that did not carry a T7 polymerase despite the fact that transcription from plasmid pNL1A1 was driven by a T7 promoter. This level of CobS activity was approximately sevenfold higher than that obtained by inducing the chromosomal *cobUST* genes. Spheroplasts of this strain were lysed by osmotic shock and membranes were isolated by centrifugation (144 000 g). Inner and outer membranes were separated by isopycnic ultracentrifugation as described by Osborn & Munson (1974). Fractions (250 µl each) were collected after piercing the bottom of the polyallomer centrifuge tube (Beckman) with a needle. The *A₂₈₀* was measured for every fraction using a quartz microtitre plate and SpectraMAX Plus spectrophotometer (Molecular Devices). Fractions were assayed for Sdh and CobS activities. Western blot analysis employing anti-CobS antibodies (see below) was also used to identify the cellular location of CobS. The fractionation procedure was performed twice.

**Cell fractionation of *M. thermoautotrophicum* strain ΔH.** Frozen cell pellets (3 g) were thawed and resuspended in 10 ml 50 mM MOPS/NaOH buffer pH 7 containing 10 mM MgCl₂. Cells were disrupted using a French pressure cell, and cell debris was removed by centrifugation (10 000 g, 30 min). Cleared cell-free extract was subjected to ultracentrifugation at 125 000 g for 3 h. Pellets were resuspended in MOPS buffer and centrifuged again under the same conditions for an additional 2 h.

**Generation of rabbit anti-CobS antibodies and Western blot analysis.** Polyclonal antibodies were raised against a synthetic 25 amino acid peptide of CobS (DTCDGIFSARRERMLIEIMRDSRLG) generated at the Peptide Synthesis Facility at the Biotechnology Center of the University of Wisconsin-Madison. Four peptides were conjugated to a branching lysine core molecule to form a multiple antigenic peptide (Posnett *et al.*, 1988). Antibodies were elicited in a New Zealand White rabbit at the Animal Care Unit at the Medical School of the University of Wisconsin-Madison. Serum from the sixth bleed was pre-cleared (Suh, 1994) using strain JE877 (Table 1). Western blot analysis was performed as described previously (Rondon & Escalante-Semerena, 1997), using a 1:10 000 dilution of CobS antibody.

**RESULTS**

**Complementation of function by bacterial and archaeal CobS enzymes**

All *cobS*-containing plasmids used in the studies described below except the plasmids encoding CobS–PhoA and CobS–LacZ protein fusions were tested for their ability to correct the cobalamin biosynthetic defect of strain JE1805 (*cobS*, Table 1). Plasmids pNL1A1, pCOBS4, pCOBS6 and pCOBS9 encoded functional CobS proteins that restored AdoCbl biosynthesis, thus allowing strain JE1805 to grow on minimal medium supplemented with (CN)₂Cbl (data not shown). The CobS protein encoded by pCOBS10 lacked the first 35 amino acids, and this plasmid failed to correct the cobalamin auxotrophy of strain JE1805. Plasmid pCOBS16, carrying the *M. thermoautotrophicum* strain ΔH orthologue of *cobS*, also corrected the cobalamin auxotrophy of strain JE1805 (Fig. 2). As expected, complementation was not observed with empty cloning vector pT7-7.

*Fig. 2.* Archaeal CobS function compensates for the lack of bacterial CobS function. The recombination-deficient strain JE1805 (*cbiD cobS recA*; □) was used as recipient of plasmids pNL1A1 (*S. enterica cobUST*⁺; ○), pCOBS4 (*S. enterica cobS*⁺; ◊) or pCOBS16 (*M. thermoautotrophicum cobS*⁺; ▲). Growth conditions were as described in Methods. VOC, vector-only control (plasmid pT7-7); Cbi, dicyanocobamin; Obl, cyanocobamin.

**Other methods.** Protein concentrations were determined as described by Kunitz (1952). Non-radioactive DNA sequencing of plasmid constructs was performed using the ABI PRISM BigDye terminator cycle sequencing kit v3.1 (PerkinElmer Life Sciences) according to the manufacturer’s instructions. DNA sequences were determined at the Biotechnology Center at the University of Wisconsin-Madison.
Expression of the \textit{cobS} gene in \textit{E. coli}

Overexpression of CobS using the inducible T7 polymerase promoter was attempted to facilitate biochemical analysis of the protein. \textit{E. coli} BL21(DE3) carrying plasmid pCOBS4 or plasmid pT7-7 was grown on M9 minimal medium, and gene expression was induced with IPTG. Protein patterns of cells grown under these conditions were analysed by SDS-PAGE and Coomassie blue staining. Analysis of a gel containing samples taken prior to (Fig. 3a, lane 3), and 2 h after induction (Fig. 3a, lane 4) revealed a protein with an approximate mass of 26 kDa at an increased level in the strain carrying pCOBS4. The level of this protein, however, increased only slightly upon induction with IPTG, and its level was not appreciably different in samples taken at 4 and 24 h after induction (data not shown). Expression of the 26 kDa protein in the absence of IPTG indicated that residual level of \textit{cobS} transcription from pCBOS4 was sufficient to elicit its synthesis. Restoration of AdoCbl synthesis in a \textit{S. enterica} \textit{cobS} strain by pCOBS4 in the absence of exogenous IPTG (Fig. 2) provided evidence for residual transcription of \textit{cobS} from this plasmid. The molecular mass of the overproduced protein was consistent with that predicted by the nucleotide sequence of the \textit{cobS} gene (26-3 kDa). After four chromatographic steps (anion exchange, hydroxyapatite, gel permeation and B12-affinity chromatography) the overproduced protein was subjected to N-terminal analysis. The sequence obtained was G\text{IFS\textit{R\textit{F\textit{AD\textit{IVN\textit{A\textit{N}}}}}}, which matched that of the phage stress protein A (PspA) protein (GenBank accession no. C64879). The predicted mass of the PspA protein was 25-5 kDa. Synthesis of PspA protein in \textit{E. coli} has been reported to correlate with membrane stress in this bacterium (Model \textit{et al.}, 1997). The identity of the 26 kDa protein was confirmed in vivo to be PspA by expressing the \textit{cobS} gene from plasmid pCOBS4 in strain BL21(DE3) \textit{Dpsp\textsubscript{+}} and comparing the protein pattern to that of strain BL21(DE3) \textit{psp\textsubscript{+}/pCOBS4} (Fig. 3b, compare lanes 7 and 8).

CobS is a membrane protein

Accumulation of the PspA protein upon overexpression of the \textit{cobS} gene suggested that the CobS protein perturbed the architecture or function of the cell membrane. To determine the location of the cobalamin synthase enzyme, cell membranes were isolated by ultracentrifugation. The results of these experiments are summarized in Table 2. Ultracentrifugation of S30 preparations of strain TR6583 (\textit{cobS\textsuperscript{+}}) at 100 000 \textit{g} produced a pellet containing 83 \% of the total cobalamin synthase activity in the crude cell-free extract (0-342 U). As expected, at this centrifugal force the pellet of material obtained contained a considerable amount of cell membrane and ribosomes (Tai & Kaplan, 1985). Cell membrane and ribosomes were resolved by

\begin{table}[h]
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\caption{Cobalamin synthase activity of various ribosome preparations}
\begin{tabular}{|l|c|c|}
\hline
 & \textbf{Activity (total units)} & \\
 & \textbf{Cobalamin synthase} & \textbf{Succinate dehydrogenase} \\
\hline
\textbf{Crude ribosome preparation} & & \\
S30 (starting material) & 0-383 & NA \\
S100 & 0-072 & NA \\
Ribosome pellet & 0-342 & NA \\
\hline
\textbf{Salt-sucrose ribosome preparation} & & \\
S30 (starting material) & 0-123 & 32-5 \\
1. Salt + sucrose cushion & & \\
S100 & 0-195 & 35-6 \\
Ribosome pellet & 0-005 & ND \\
2. Sucrose cushion & & \\
S100 & 0-120 & 31-8 \\
Ribosome pellet & 0-038 & 8-3 \\
\hline
\end{tabular}
\end{table}

NA, Not assayed; ND, none detected.
centrifuging the S30 extract through a salt and sucrose cushion. Under these conditions, the pellet contained ribosomes and only 2.5% of the total cobalamin synthase activity. Omission of salt from this procedure produced a pellet containing 24% of the cobalamin synthase activity (0.038 U). This ribosome pellet also contained 21% of total succinate dehydrogenase (EC 1.3.99.1) activity in the extract. Since Sdh is an inner-membrane protein, this result indicated that sedimentation of the inner membrane was partially retarded by the sucrose cushion.

We took advantage of the differences in buoyancy between ribosomes and membranes, and used isopycnic density ultracentrifugation to separate inner and outer membranes from each other and from ribosomes. The results of the fractionation of these components in strain JE5896 are presented in Fig. 4(a). A280 was used to visualize the positions of the outer membrane (high density), the inner membrane (two peaks of low density) and any unresolved envelope (intermediate density) (Osborn & Munson, 1974). Under the conditions employed, ribosomes were expected to form a pellet at the bottom of the tube (Spedding, 1990). In this profile, cobalamin synthase activity co-eluted with the succinate dehydrogenase activity in the inner membrane fraction. The presence of the CobS protein in these fractions was further supported by Western blot analysis (Fig. 4b).

Membrane topology of CobS as determined by analysis of CobS–PhoA and CobS–LacZ protein fusions

The program TmPred, available through the European Molecular Biology Network (Hofmann & Stoffel, 1993), was used to analyse the hydropathy of the predicted CobS amino acid sequence. The predicted topology model generated by TmPred (Fig. 5a), was used to guide the selection of sites on the CobS protein to which PhoA and LacZ proteins would be fused. A summary of the results is presented in Table 3 and a modified model of the CobS membrane topology based on the results obtained with LacZ and PhoA fusions is shown in Fig. 5(b). In this model, residues conserved in CobS orthologues (Fig. 5) face the cytoplasm. Background levels of β-galactosidase and alkaline phosphatase activities were determined with cells harbouring the parent plasmid pCOBS6 without a reporter fused to CobS. No β-galactosidase was detectable in the control strain, while seven molecules of active alkaline phosphatase-like activity were measured per cell in the same strain.

Residues exposed to the cytoplasm. The TmPred model predicted that amino acid residues 28, 101, 179 and 243 would be exposed to the cytoplasm. Alkaline phosphatase and β-galactosidase activities measured in cells containing CobS28–, CobS101– or CobS179–LacZ, or CobS28–, CobS101– or CobS179–PhoA protein fusions supported the predictions for these three locations, even though the level of alkaline phosphatase measured in cells carrying the plasmid encoding the CobS28–PhoA fusion protein was twofold over background. The data indicated that residue R179 was accessible to the cytoplasm. Unlike what was predicted by the model, alkaline phosphatase and β-galactosidase activities in cells containing CobS243–LacZ or CobS243–PhoA protein fusions suggested that the C-terminus of CobS was most likely embedded in the membrane.

Residues exposed to the periplasmic space. The model predicted that three loops of the CobS protein would be exposed to the periplasm. Amino acid residues 131 and 199 were chosen as fusion sites. Cells containing CobS199–LacZ or CobS199–PhoA fusion proteins had low levels of β-galactosidase activity per cell but the number of active molecules of alkaline phosphatase indicated that these residues were exposed to the periplasm (Table 3). Surprisingly, cells making CobS131–LacZ fusions had 16-fold higher-than-background activities of β-galactosidase. This unexpected result was difficult to explain, especially in light of the strong activity level measured in cells making the corresponding CobS131–PhoA fusion. The TmPred model was used to predict that amino acid residues 28, 101, 179 and 243 would be exposed to the cytoplasm. Alkaline phosphatase and β-galactosidase activities measured in cells containing CobS28–, CobS101– or CobS179–LacZ, or CobS28–, CobS101– or CobS179–PhoA protein fusions supported the predictions for these three locations, even though the level of alkaline phosphatase measured in cells carrying the plasmid encoding the CobS28–PhoA fusion protein was twofold over background. The data indicated that residue R179 was accessible to the cytoplasm. Unlike what was predicted by the model, alkaline phosphatase and β-galactosidase activities in cells containing CobS243–LacZ or CobS243–PhoA protein fusions suggested that the C-terminus of CobS was most likely embedded in the membrane.
fusion protein. The PhoA data for this location were consistent with the predicted periplasmic exposure of residue L131 to the periplasm.

Residues of CobS embedded in the membrane. The low level of PhoA activity measured in cells making CobS55–PhoA protein suggested that residue L55 was likely to be embedded in the membrane, but the PhoA protein fused to it could still reach the periplasmic space and become active. Alkaline phosphatase and β-galactosidase activities in cells making CobS185– and CobS243–LacZ/PhoA fusion proteins were consistent with these residues being embedded in the membrane. Residue L196 was also placed within the membrane. The high level of PhoA activity of the CobS196–PhoA fusion protein was explained by the closeness of L196 to the periplasm. A similar argument was made to interpret the data obtained with CobS179–LacZ protein. In the model shown in Fig. 5(b), residue R179 is located very close to the cytosol, but still within the membrane. Activity measurements of fusions to residues L74 and L157 were ambiguous, but their location within the membrane would be consistent with the model. Activity measurements with the CobS115 protein fusions suggested that residue L115 was embedded in the membrane but sufficiently close to the cytosol for LacZ to reach it and become active.

The CobS orthologue from the archaeon

*M. thermoautothrophicum* strain ΔH is also a membrane protein

As mentioned above, the *cobS* gene of the methanogenic archaeon *M. thermoautothrophicum* strain ΔH compensated for the lack of CobS in *S. enterica* *cobS* strains during B12-dependent growth (Fig. 2). This result suggested that the archaeal protein was localized to the membrane. To investigate this possibility, spheroplasts of strain JE6002 (*S. enterica* *cobS*/*M. thermoautothrophicum* pCOBS16 *cobS*+) were obtained. Total cell membranes of strain JE6002 were isolated following lysis by osmotic shock. This membrane preparation contained $3.5 \times 10^{-3}$ U cobalamin synthase activity per mg protein when assayed at 50°C. An S30 extract of strain JE6002 had a 10-fold lower specific activity ($3.5 \times 10^{-4}$ U per mg protein), indicating that the protein accumulated in the membrane. The activity of the *M. thermoautothrophicum* cobS orthologue was twofold lower when assayed at 37°C, the temperature at which it complemented a *S. enterica* cobS mutant.

We used ultracentrifugation to fractionate *M. thermoautothrophicum* strain ΔH cell-free extracts (Gärtner *et al*., 1993; Heiden *et al*., 1994). Ninety percent of the total cobalamin synthase activity was detected in the washed pellet, distributed as 64% in the tight pellet and 26% in the gelatinous pellet. These pellets are both considered to be part of the membrane fraction (Fischer *et al*., 1992; Heiden *et al*., 1994). The specific activities for these pellets were $2.5 \times 10^{-4}$ and $3.8 \times 10^{-4}$ U per mg protein, respectively, and were more than 10-fold higher than that found for the supernatant ($2.2 \times 10^{-5}$ U per mg protein).

**DISCUSSION**

**Implications of a membrane-associated cobalamin synthase activity for de novo AdoCba synthesis and corrinoid salvaging**

Our results from ultracentrifugation and topology experiments support the conclusion that the cobalamin synthase protein is localized to the membrane of the bacterium *Salmonella enterica* and the archaeon *Methanobacterium thermoautothrophicum* strain ΔH. We used bioinformatics tools available online (http://megaman.ucsd.edu) to align
putative cobS orthologues from eight archaea (M. thermoautotrophicum, Methanococcus jannaschii, Archaeoglobus fulgidus, Pyrococcus horikoshii, Pyrodiction abyssi, Thio-
bacillus acidophilum and Thermoplasma volcanium) and six bacteria (E. coli, Vibrio cholerae, Mesorhizobium loti, Synechocystis sp., Mycobacterium tuberculosis and Bacillus halodurans). All of the above-mentioned CobS orthologues showed extensive, end-to-end regions of hydrophobicity (data not shown), suggesting a strong, unidentified selective pressure to maintain the late steps of AdoCba biosynthesis in close association with the cell membrane. The topological analysis of CobS performed using protein fusions to alkaline phosphatase or β-galactosidase as reporters was not unambiguous for all the fusions analysed; hence detailed knowledge of the association of CobS to the membrane is still incomplete. However, as a whole, the data provide good evidence to conclude that CobS is an integral membrane protein. The most intriguing question raised by these results is: why should coenzyme B12 synthesis be associated with the membrane? The CobS protein is not the only membrane-associated enzyme of the pathway. Interestingly, the cobinamide-phosphate synthase (CbiB) enzyme is also an integral membrane protein (C. L. Zayas & J. C. Escalante-Semerena, unpublished results). The CbiB enzyme catalyses a step of the pathway that is key to salvaging of cobyric acid from the environment. Whatever the reason for the association of these two enzymes with the membrane, it is not unique to Salmonella enterica. To date, all of the genome sequences of B12-producing prokaryotes that have CobS or CbiB are predicted to be very hydrophobic proteins, and hence likely to be associated with the cell membrane. Here we provided support for this idea by looking at the location of one CobS orthologue in the methanogenic archaeon M. thermoautotrophicum strain ΔH. Answers to the question raised above will provide insights into the strategy prokaryotes need to use to successfully synthesize this important coenzyme.

**Is there a connection between membrane stress and high levels of CobS protein?**

In many instances protein overproduction reveals stress responses that are consistent with physiological strategies aimed at maintaining some proteins at low levels in the wild-type strain. It is too early to tell why CobS cannot be overproduced. So far, it appears that CobS overproduction stresses the cell membrane, affecting its structure and/or function. This idea is indirectly supported by the overproduction of the membrane-stress indicator protein PspA that results from cobS overexpression. Physiological responses associated with membrane stress may be a mechanism used by S. enterica for maintaining cobamide biosynthesis at low levels.

**Conflict with previously reported work**

The localization of the CobS enzyme to the cell membrane of S. enterica conflicts with results from previous work in E. coli, in which cobalamin synthase activity was reported to be associated with the ribosomal L18 protein (Pezacka & Walerych, 1981). In considering the physiological relevance of the putative cobalamin synthase activity of the L18 protein one must bear in mind that: (i) the L18 protein failed to use x-ribazole and x-ribazole phosphate, the documented substrates of cobalamin synthase (Walerych et al., 1968); (ii) the work on the L18 protein predates the identification of the cob genes in any prokaryote, and since the discovery of the cob genes, cobalamin synthase activity has been shown to be encoded by the cobS gene of...
S. enterica and the cobV gene of P. denitrificans (Cameron et al., 1991; Maggio-Hall & Escalante-Semerena, 1999); (iii) cobS mutant strains of S. enterica lack cobalamin synthase activity and are unable to convert AdoCbi to AdoCbl (Jeter et al., 1984; O’Toole et al., 1993). If the L18 protein had a physiologically relevant cobalamin synthase activity, no phenotype would be observed; (iv) in spite of the use of an extremely sensitive bioassay, cobalamin synthase activity was not detectable in cell-free extracts of strains carrying a deletion of the cobS gene (Maggio-Hall & Escalante-Semerena, 1999; O’Toole et al., 1993) and this work, it is concluded that the cobalamin synthase activity of the CobS protein is essential for AdoCbl synthesis, and is associated with the inner membrane in this bacterium, and probably in all corrinoid-producing prokaryotes.

Although the work reported here did not investigate the putative cobalamin synthase activity of the L18 protein in S. enterica, on the basis of previously reported genetic and biochemical evidence (Maggio-Hall & Escalante-Semerena, 1999; O’Toole et al., 1993) and this work, it is concluded that the cobalamin synthase activity of the CobS protein is essential for AdoCbl synthesis, and is associated with the inner membrane in this bacterium, and probably in all corrinoid-producing prokaryotes.

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