Biochemical evidence that the *pduS* gene encodes a bifunctional cobalamin reductase

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*Salmonella enterica* degrades 1,2-propanediol (1,2-PD) by a pathway that requires coenzyme B12 (adenosylcobalamin; AdoCbl). The genes specifically involved in 1,2-PD utilization (*pdu*) are found in a large contiguous cluster, the *pdu* locus. Earlier studies have indicated that this locus includes genes for the conversion of vitamin B12 (cyanocobalamin; CNCbl) to AdoCbl and that the *pduO* gene encodes an ATP:cob(I)alamin adenosyltransferase which catalyses the terminal step of this process. Here, *in vitro* evidence is presented that the *pduS* gene encodes a bifunctional cobalamin reductase that catalyses two reductive steps needed for the conversion of CNCbl into AdoCbl. The PduS enzyme was produced in high levels in *Escherichia coli*. Enzyme assays showed that cell extracts from the PduS expression strain reduced cob(III)alamin (hydroxycobalamin) to cob(II)alamin at a rate of 91 nmol min\(^{-1}\) mg\(^{-1}\) and cob(II)alamin to cob(I)alamin at a rate of 7.8 nmol min\(^{-1}\) mg\(^{-1}\). In contrast, control extracts had only 9.9 nmol min\(^{-1}\) mg\(^{-1}\) cob(III)alamin reductase activity and no detectable cob(II)alamin reductase activity. Thus, these results indicated that the PduS enzyme is a bifunctional cobalamin reductase. Enzyme assays also showed that the PduS enzyme reduced cob(II)alamin to cob(I)alamin for conversion into AdoCbl by purified PduO adenosyltransferase. Moreover, studies in which iodoacetate was used as a chemical trap for cob(I)alamin indicated that the PduS and PduO enzymes physically interact and that cob(I)alamin is sequestered during the conversion of cob(II)alamin to AdoCbl by these two enzymes. This is likely to be important physiologically, since cob(I)alamin is extremely reactive and would need to be protected from unproductive by-reactions. Lastly, bioinformatic analyses showed that the PduS enzyme is unrelated in amino acid sequence to enzymes of known function currently present in GenBank. Hence, results indicate that the PduS enzyme represents a new class of cobalamin reductase.

**INTRODUCTION**

*Salmonella enterica* degrades 1,2-propanediol (1,2-PD) in a coenzyme B12 (adenosylcobalamin; AdoCbl)-dependent fashion (Jeter, 1990). 1,2-PD is a major product of the fermentation of the common plant sugars rhamnose and fucose and is thought to be an important carbon source in anaerobic environments such as the large intestines of higher animals (Obradors *et al.*, 1988; Toraya *et al.*, 1980). Virtually all natural isolates of *Salmonella* degrade 1,2-PD and *in vivo* studies with mice have suggested that the degradation of this small molecule may be important for the interaction of *S. enterica* with its host organisms (Conner *et al.*, 1998; Heithoff *et al.*, 1999).

The pathway of 1,2-PD degradation begins with the conversion of 1,2-PD to propionaldehyde by AdoCbl-dependent diol dehydratase (Bobik *et al.*, 1997; Obradors *et al.*, 1988; Toraya *et al.*, 1979). Subsequently, propionaldehyde is converted to propanol and propionate by alcohol dehydrogenase, CoA-dependent propionaldehyde dehydrogenase, phosphotransacetylase and propionate kinase (Obradors *et al.*, 1988; Toraya *et al.*, 1979). This pathway generates one ATP, an electron sink for the regeneration of NAD and an intermediate (propionyl-CoA) that can serve as a carbon and energy source via the methylcitrate pathway (Horswill & Escalante-Semerena, 1997).

Although the pathway of 1,2-PD degradation appears quite straightforward, the catabolism of this small molecule is in fact a very elaborate process (Bobik *et al.*, 1999; Havemann *et al.*, 2002; Havemann & Bobik, 2003). Recent studies have shown that a polyhedral body is involved in AdoCbl-dependent 1,2-PD degradation by *S. enterica* (Bobik *et al.*, 1999; Havemann *et al.*, 2002; Havemann & Bobik, 2003). These polyhedra are 100–150 nm across,
consist of a proteinaceous shell and interior and are composed of at least 15 different polypeptides, including four enzymes essential for 1,2-PD degradation (Havemann & Bobik, 2003). While our understanding of these unusual structures is still relatively limited, studies conducted so far indicate that their role is to mitigate the toxicity of propionaldehyde, an obligatory intermediate of the 1,2-PD catabolic pathway (Havemann et al., 2002; Havemann & Bobik, 2003; Leal et al., 2003a).

Adding to the complexity of 1,2-PD degradation is the fact that this process requires systems for the acquisition and maintenance of AdoCbl. S. enterica can obtain AdoCbl by de novo synthesis and by uptake from the environment (Roth et al., 1996). In addition, S. enterica can transport corrinoid precursors such as hydroxycobalamin (HOCbl) and cyanocobalamin (CNClb; vitamin B12) and convert these compounds to AdoCbl (Roth et al., 1996). The de novo synthesis of AdoCbl occurs only under anoxic conditions, but the use of exogenous AdoCbl or complex precursors proceeds both aerobically and anaerobically. The use of corrinoid precursors requires addition of the appropriate upper ligand to the central cobalt atom of the corrin ring, which requires several enzymatic steps (Huennekens et al., 1982; Vitols et al., 1965; Weissbach et al., 1961) (Fig. 1). First, CNClb is converted to HOCbl by cobalamin β-ligand transferase (Brady & Barker, 1961; Pezacka et al., 1990; Pezacka, 1993; Watanabe et al., 1987; Weissbach et al., 1962). Next, two successive one-electron reductions of the central cobalt atom of HOCbl lead to the formation of cob(II)alamin and cob(I)alamin (Brady & Barker, 1961; Brady et al., 1962; Fonseca & Escalante-Semerena, 2000, 2001; Fujii & Huennekens, 1974; Fujii et al., 1977; Pezacka, 1993; Walker et al., 1969; Watanabe et al., 1987, 1993, 1996; Weissbach et al., 1961). These reactions are catalysed by cob(III)alamin reductase and cob(II)alamin reductase, respectively. Lastly, ATP : cob(I)alamin adenosyltransferase catalyses the transfer of a 5′-deoxyadenosyl group from ATP to cob(I)alamin, forming AdoCbl (Brady et al., 1962; Debussche et al., 1991; Dobson et al., 2002; Johnson et al., 2001; Leal et al., 2003b; Peterkofsky et al., 1961; Suh & Escalante-Semerena, 1995; Vitols et al., 1965). Interestingly, this pathway has a dual function. In addition to its role in the assimilation of exogenous corrinoid compounds, it is also needed for recycling HOCbl generated endogenously from the breakdown of AdoCbl, which is unstable during catalysis (Toraya & Mori, 1999; Toraya, 2000).

The genetics of 1,2-PD degradation reflects the complexity of this process. In S. enterica, the genes specifically involved in 1,2-PD utilization (pdu) are found in a single, large, contiguous cluster, the pdu locus (Bobik et al., 1999; Jeter, 1990). Included are the pocR and pduU genes (which encode a positive transcriptional regulator and a 1,2-PD diffusion facilitator, respectively) (Bobik et al., 1992; Chen et al., 1995) as well as the adjacent, but divergently transcribed, pdu operon, which includes 21 additional genes (Bobik et al., 1999; Jeter, 1990). Of these 21 genes, six encode enzymes of the 1,2-PD degradative pathway (pduCDPEQW) (Bobik et al., 1997, 1999; Leal et al., 2003a) and seven are thought to encode structural proteins required for polyhedral body formation (pduABJKNTU) (Bobik et al., 1999; Havemann et al., 2002; Havemann & Bobik, 2003). Based on high sequence similarity, the pduGH genes are likely to encode a protein required for reactivation of diol dehydratase following the breakdown of AdoCbl (Bobik et al., 1999). The pduO gene was shown to encode an ATP : cob(I)alamin adenosyltransferase used for the conversion of complex precursors into AdoCbl (Johnson et al., 2001). Finally, five pdu genes are of unknown function (pduLMNSV) and are unrelated to genes of known function found in GenBank (Bobik et al., 1999).

Earlier studies of 1,2-PD degradation have indicated that the pdu operon includes genes for the conversion of CNClb to AdoCbl, but so far only the pduO gene has been shown to be involved in this process (Johnson et al., 2001). Recent bioinformatic analyses indicated that the PduS protein has iron–sulfur and NAD-binding motifs, suggesting that this enzyme might be involved in cobalamin reduction (unpublished data). In this report, we present in vitro evidence that the PduS enzyme is a bifunctional cobalamin reductase used for the conversion of CNClb into AdoCbl.

METHODS

Chemicals and reagents. AdoCbl, CNClb and HOCbl were from Sigma. IPTG was from Diagnostic Chemicals Ltd. Restriction enzymes and T4 DNA ligase were from New England Biolabs. Other chemicals were from Fisher Scientific.

Bacterial strains and media. The bacterial strains used in this study were S. enterica serovar Typhimurium LT2 (formerly S. typhimurium LT2), Escherichia coli DH5α and E. coli BL21 DE3 RIL (Strategene). LB (Luria–Bertani) medium was the rich medium used (Miller, 1972).

General molecular methods. Agarose gel electrophoresis was performed as described by Maniatis et al. (1982). Plasmid DNA was purified by the alkaline lysis procedure (Maniatis et al., 1982) or by using Qiagen products according to the manufacturer’s instructions. Following restriction digestion or PCR amplification, DNA was purified using Qiagen PCR purification and gel extraction kits or by using phenol/chloroform extraction followed by ethanol precipitation (Maniatis et al., 1982). Restriction digests were carried out

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**Fig. 1.** Proposed pathway for the conversion of CNClb to AdoCbl. This pathway has a dual function: it is needed for the assimilation of exogenous corrinoids such as CNClb and HOCbl, as well as for the recycling of HOCbl generated endogenously from the breakdown of AdoCbl during catalysis. The cobalt atom of HOCbl is in the 3+ oxidation state; hence, HOCbl is a substrate for cob(III)alamin reductase.
using standard protocols (Maniatis et al., 1982). For ligation of DNA fragments, T4 DNA ligase was used according to the manufacturer’s instructions. Electroporation was used for bacterial transformation. A Gene Pulser (Bio-Rad) was used according to the manufacturer’s instructions and at the following settings: capacitance, 25 μF; capacitance extender, 250 μF; pulse controller, 200 μF; voltage, 2-5 kV. LB medium containing the appropriate antibiotic(s) was used to select for transformed cells and, prior to the analysis of transformants, pure cultures were prepared.

**General protein methods.** PAGE was performed using Bio-Rad Redigel and Mini-protein II electrophoresis cells. PAGE was run at 200 V (constant voltage) for 45 min using a Bio-Rad Powerpack 300. Following gel electrophoresis, Coomassie brilliant blue R-250 was used to stain proteins. The protein concentration of solutions was determined using Bio-Rad protein assay reagent according to the manufacturer’s instructions with BSA as the standard.

**DNA sequencing and analysis.** DNA sequencing was carried out by the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility using Applied Biosystems Inc. automated sequencing equipment (Perkin Elmer). The template for DNA sequencing was plasmid DNA purified using Qiagen tip 100 columns. BLAST software was used for sequence similarity searches (Altschul et al., 1990, 1997).

**Cloning the pduS gene for high-level expression.** PCR was used to amplify the pduS coding sequence for cloning into a modified T7 expression plasmid, pTA925 (Johnson et al., 2001). The Pfu polymerase was used because of its high fidelity of DNA replication. Template pMG52 was used with primers 5′-GGAATTCAATCTTTGAACACACCCATACAAAGC-3′ (forward) and 5′-GGAATT-CAAGCTTTGGTTAACCTCTTACAACAGTG-3′ (reverse). These primers introduced the BglII and HindIII restriction sites that were used to clone the pduS coding sequence into pTA925. Ligation mixtures were used to transform E. coli strain DH5α and transformants were selected by plating on LB agar supplemented with 25 μg kanamycin ml⁻¹. Plasmid DNA isolated from selected transformants was analysed by restriction digestion and DNA sequencing. One plasmid that contained a pduS insert with the expected DNA sequence (pAP253) was introduced into expression strain E. coli BL21 DE3 RIL by electroporation and the resulting strain (BE311) was used for production of recombinant PduS protein.

**Growth of the PduS expression strain and preparation of cell extracts.** E. coli strain BE311 was grown in 500 ml LB kanamycin (25 μg ml⁻¹) broth incubated at 20°C with shaking at 275 r.p.m. in a 1 litre baffled Erlenmeyer flask. Cells were grown to an OD₆₀₀ of 0.6–0.8. Next, expression of the pduS gene was induced by the addition of IPTG to a concentration of 0.5 mM. Cells were incubated for an additional 8–12 h and harvested by centrifugation at 6690 g for 10 min using a Beckman JLA-10.500 rotor. Following centrifugation, all subsequent procedures were carried out using anaerobic procedures. Two grams of cells (wet weight) were resuspended in 3 ml of a solution containing 50 mM sodium phosphate pH 7, 50 mM KCl, 1 mM DTT and 0.24 mg of the protease inhibitor Pefabloc ml⁻¹ (Pentapharm). Cells were broken using a French pressure cell (SLM Aminco) at 20000 p.s.i. Soluble proteins were separated from inclusion bodies by centrifugation of cell extracts at 31000 g for 30 min using a Beckman JA-20 rotor. The supernatant obtained was the soluble cell extract used for further studies. The pellet was treated with Bacterial Protein Extraction Reagent II (Pierce Biotechnology) according to the manufacturer’s instructions and under anaerobic conditions to obtain the purified inclusion bodies used for further analyses. Control strain BE119 (E. coli BL21 DE3 RIL/pTA925-no insert) was grown in parallel with expression strain BE311 and cell extracts of this strain were similarly prepared.

**Cob(III)alamin reductase assays.** Cob(III)alamin reductase assays were a modification of a previously described method (Watanabe et al., 1987). Assay mixtures contained 50 mM CHES/NaOH (pH 8.5), 1.6 mM KH₂PO₄, 2.8 mM MgCl₂, 0.5 mM NADH and 100 μM HOCbl. The total assay volume was 2 ml and assays were carried out using strict anaerobic precautions. Inside an anoxic chamber (Coy Laboratory Products), assay components were dispensed into modified glass cuvettes, sealed with 13 mm grey butyl rubber stops and aluminium crimp seals, removed from the chamber and flushed with N₂ for 30 s (Johnson et al., 2001). Cuvettes were placed in a 37°C water bath for 5 min and reactions were initiated by adding a source of enzyme or a particular assay component using the following procedures to minimize the introduction of oxygen. The assay component to be added was placed within a sealed serum vial and flushed with N₂ for 2 min. Additions were made using a Hamilton syringe that had been flushed with anoxic water just prior to use. Reaction rates were determined by monitoring the decrease in absorbance at 525 nm and using Δ₅₂₅ = 4.9 mM⁻¹ cm⁻¹ for calculations.

**Linked cob(II)alamin reductase assays.** For the measurement of cob(II)alamin reductase activity, a linked assay with the PduO adenosyltransferase was used (Fig. 2). The basis of this assay is that the PduO enzyme converts cob(II)alamin to AdoCbl, which occurs with a concomitant increase in absorbance at 525 nm (Δ₅₂₅ = 4.8 mM⁻¹ cm⁻¹). PduO is specific for cob(II)alamin and does not react with cob(II)alamin (Johnson et al., 2001). Anaerobic precautions were used as described above for cob(III)alamin reductase assays. Reaction mixtures contained 50 mM CHES/NaOH (pH 8.5), 1.6 mM KH₂PO₄, 2.8 mM MgCl₂, 100 μM cob(II)alamin, 0.4 mM ATP, 1 mM NADH, 120 μg purified PduO adenosyltransferase and a source of cob(II)alamin reductase. Assays were carried out at 37°C and reactions were initiated by adding a source of enzyme or a particular assay component as indicated in the text. Cob(II)alamin was generated by exposing a 10 mM AdoCbl stock solution to a 150 W incandescent light at a distance of 20 cm for 10–20 min under anaerobic conditions at room temperature. Purified PduO adenosyltransferase was obtained from recombinant E. coli strain BE118 (Johnson et al., 2001; unpublished data).

**Measurement of ‘free’ cob(II)alamin using iodoacetate.** Anoxic stock solutions of iodoacetate (40 mM) were prepared and used the same day. The stock solution was shielded from light using aluminium foil and was added to assay mixtures just before the initiation of reactions using the anoxic procedures described above for cob(II)alamin reductase assays. Iodoacetate reacts rapidly and quantitatively with cob(II)alamin to form carboxymethyl-cobalamin (CM-Cbl). This occurs with a concomitant increase in absorbance at 525 nm, which allows quantification of the cob(II)alamin formed. Cobalamin reduction by PduS

![Fig. 2. Linked cob(II)alamin reductase assay. In this assay, the PduO adenosyltransferase is used to convert cob(II)alamin to AdoCbl. This occurs with an increase in absorbance at 525 nm, which allows quantification of the cob(II)alamin formed. The PduO enzyme is specific for cob(II)alamin and does not react with cob(II)alamin (Johnson et al., 2001).](https://www.microbiologyresearch.org)
HPLC analysis of AdoCbl, CM-Cbl and HOCbl. Reverse-phase HPLC was used to separate and quantify HOCbl, AdoCbl and CM-Cbl. A NovaPak C18 column (3×9×150 mm) equipped with a C18 Sentry guard column was used for the separation (Waters). Samples (200 μl) were loaded onto the column and eluted with a 30 min linear gradient of 10 to 90% methanol in 100 mM sodium acetate (pH 4.6) at a flow rate of 1 ml min⁻¹. The absorbance of effluent was monitored at 365 nm and analytes were quantified by comparison of peak areas to a standard curve. The CM-Cbl standard was prepared by incubating a solution of 200 mM Tris/HCl pH 8.0, 1.6 mM potassium phosphate, 2.8 mM MgCl₂, 100 mM KCl, 100 μM cob(II)alamin, 1 mM DTT, 50 μM FAD and 0.4 mM iodoacetate at 37°C for 1 h under strictly anaerobic conditions, which resulted in quantitative conversion of cob(II)alamin to CM-Cbl (data not shown).

RESULTS

High-level production of the PduS protein

E. coli strain BE311 was constructed to produce high levels of recombinant PduS protein. Protein production by this strain as well as control strain BE119 was analysed by SDS-PAGE (Fig. 3). For expression strain BE311, relatively large amounts of protein with a molecular mass near that predicted for the PduS protein (48.4 kDa) were found in the soluble fraction of cell extracts. In contrast, relatively little protein near 48 kDa was found in extracts from control strain BE119. Similar results were obtained when the inclusion body fractions of cell extracts were analysed by SDS-PAGE (not shown). These results indicated that the majority of the observed 48-kDa protein expressed by BE311 was recombinant PduS.

The PduS protein has cob(III)alamin reductase activity

The cell extracts used for SDS-PAGE (Fig. 3) were assayed for cob(III)alamin (HOCbl) reductase activity using the continuous spectrophotometric assay described in Methods. Based on the mean of three trials, cell extracts from the PduS expression strain and the control contained cob(III)alamin reductase activity of 91±8 and 10±3 nmol min⁻¹ mg⁻¹, respectively. Cell extract, HOCbl and NADH were required for detectable activity and the activity of the PduS extract was reduced to the level of the control (~10±1 nmol min⁻¹ mg⁻¹) when NADPH was used as the electron donor, indicating that the PduS enzyme is specific for NADH. No activity was detected when HOCbl was replaced with CncCbl. The product of the reaction was verified to be cob(II)alamin by UV-visible spectroscopy and by the finding that exposure of the reaction product to air resulted in the formation of HOCbl (data not shown). In addition, the activity of the PduS extract was eliminated by a 30 min exposure to air, and the extract was brown in colour, as would be expected for an iron–sulfur protein. Hence, results indicated that the PduS enzyme catalysed HOCbl reduction in vitro and were consistent with bioinformatic analyses that indicated PduS was an iron–sulfur protein.

The PduS protein has cob(II)alamin reductase activity

Cell extracts from the PduS expression strain and the control strain were also assayed for the ability to reduce cob(II)alamin to cob(I)alamin [cob(II)alamin reductase activity]. The assay used was a linked assay, in which the cob(I)alamin formed was converted to AdoCbl by the PduO adenosyltransferase (Fig. 2). In assays that contained standard components, 100 μM cob(II)alamin, 0.4 mM ATP, 1 mM NADH, 120 μg purified PduO and 500 μg crude PduS extract, the rate of cob(I)alamin formation averaged 3.8±0.3 nmol min⁻¹ in three trials. No activity was detected in similar assays when the PduS extract was replaced with the control extract. For these assays, a kinetic excess of PduO was used (data not shown), and controls showed that omission of purified PduO, crude PduS, NADH, ATP or cob(II)alamin eliminated detectable cob(II)alamin reductase activity. In addition, three tests were used to verify that AdoCbl was the reaction product: (i) the UV-visible spectrum of completed reactions was characteristic of AdoCbl (Fig. 4), (ii) the reaction product co-migrated with authentic AdoCbl by reverse-phase HPLC following co-injection (not shown) and (iii) the product was photolysed by a 30 min exposure to incandescent light with the formation of cob(II)alamin (not shown).

Cob(I)alamin is sequestered during the conversion of cob(II)alamin to AdoCbl by the PduS and PduO enzymes

The studies presented above show that PduS reduces cob(II)-alamin to cob(I)alamin for the PduO adenosyltransferase.
There are two general ways in which this could occur. PduS might produce ‘free’ cob(I)alamin, which diffuses to the PduO adenosyltransferase and is converted to AdoCbl. Alternatively, PduS might interact with PduO in such a way that cob(I)alamin is sequestered during the conversion of cob(II)alamin to AdoCbl.

To test whether cob(I)alamin was sequestered or released ‘free’ in solution during the conversion of cob(II)alamin to AdoCbl by the PduS–PduO system, a chemical trap for cob(I)alamin was used. Iodoacetate, which reacts rapidly and quantitatively with cob(I)alamin to form CM-Cbl (Fonseca & Escalante-Semerena, 2000, 2001), was added to a PduS–PduO linked cob(II)alamin reductase assay in a 250-fold molar excess compared with the PduO enzyme (400 μM compared with 1.6 μM). The reaction was allowed to proceed to completion (30 min at 37 °C). CM-Cbl and AdoCbl were then resolved and quantified by reverse-phase HPLC. Results showed that 84% of the cob(I)alamin formed was converted to AdoCbl and only 16% was converted to CM-Cbl, even though iodoacetate was present in a large molar excess compared with the PduO adenosyltransferase (Table 1, row 1). This result indicated that cob(I)alamin was sequestered during the conversion of cob(II)alamin to AdoCbl by the PduS-PduO system. If cob(I)alamin had been released ‘free’ in solution, the majority should have been converted to CM-Cbl by reaction with the iodoacetate, which was present in large excess compared with PduO.

As a control, assays were performed that included PduO and iodoacetate, but in which PduS was replaced with the combination of 1 mM DTT plus 50 μM FAD to generate cob(I)alamin chemically in situ. Chemical reduction of cob(II)alamin necessarily produces ‘free’ cob(I)alamin, which must then diffuse to the adenosyltransferase before it can be converted to AdoCbl. In assays containing DTT, FAD, purified PduO and iodoacetate, only 13% of the cob(I)alamin formed was converted to AdoCbl, while 87% was converted to CM-Cbl (Table 1, row 2). This was in contrast to results obtained when PduS was used to generate cob(I)alamin for the PduO adenosyltransferase. In that case, only 16% of the cob(I)alamin formed was trapped as CM-Cbl (Table 1, row 1). If both the enzymic (PduS) and the chemical (DTT+FAD) systems had produced ‘free’ cob(I)alamin, the amount trapped as CM-Cbl should have been similar for the two systems. Hence, these results

![Fig. 4. AdoCbl is the product of linked cob(II)alamin reductase assays. UV-visible spectra of a linked cob(II)alamin reductase assay before and after incubation for 30 min at 37 °C. At t=0 (dotted line), the spectrum was that of cob(II)alamin. At t=30 min (solid line), the spectrum was characteristic of AdoCbl. Assay mixtures contained 500 μg PduS extract; other components are described in Methods.](http://mic.sgmjournals.org)

**Table 1. Cob(I)alamin is sequestered during the conversion of cob(II)alamin to AdoCbl by the PduS–PduO system**

Cob(I)alamin was generated enzymically using NADH and PduS extract (row 1) or chemically by the combination of DTT and FAD, which necessarily generates ‘free’ cob(I)alamin (row 2). In both assays, the cob(I)alamin formed should react either with iodoacetate to form CM-Cbl or with ATP (via the PduO adenosyltransferase) to form AdoCbl. If free cob(I)alamin is formed, it is expected that the majority should be converted to CM-Cbl, since iodoacetate is present in assay mixtures at a 250-fold molar excess compared with PduO. HOCbl, CM-Cbl and AdoCbl were resolved and quantified by HPLC. In the reverse-phase system used, their retention times were respectively 10-9, 13-4 and 14-3 min. The experiment was carried out twice with similar results. ND, None detected.

<table>
<thead>
<tr>
<th>Assay components varied*</th>
<th>Total corrinoid detected (nmol)</th>
<th>Cob(I)alamin converted to AdoCbl (%)†</th>
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<tbody>
<tr>
<td></td>
<td>AdoCbl</td>
<td>CM-Cbl</td>
</tr>
<tr>
<td>NADH, PduS, PduO and iodoacetate</td>
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<td>21</td>
</tr>
<tr>
<td>DTT, FAD, PduO and iodoacetate</td>
<td>24</td>
<td>160</td>
</tr>
<tr>
<td>NADH, PduS and PduO</td>
<td>128</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Reaction mixtures contained 50 mM CHES/NaOH (pH 8-5), 1-6 mM KH₂PO₄, 2-8 mM MgCl₂, 0-4 mM ATP, 200 nmol cob(II)alamin and the components indicated in the table in the following amounts: 120 μg purified PduO, 500 μg PduS extract; 1 mM NADH, 1 mM DTT, 50 μM FAD; 400 μM iodoacetate.

†To determine the percentage of cob(I)alamin converted to AdoCbl, the total cob(I)alamin formed in the assay was assumed to be equal to the amount of AdoCbl plus CM-Cbl detected [under the conditions used for analysis, the HOCbl detected was formed from the oxidation of cob(II)alamin].

To determine the percentage of cob(I)alamin converted to AdoCbl, the total cob(I)alamin formed in the assay was assumed to be equal to the amount of AdoCbl plus CM-Cbl detected [under the conditions used for analysis, the HOCl detected was formed from the oxidation of cob(II)alamin].
support the idea that the PduS–PduO system sequesters cob(1)alamin during the conversion of cob(II)alamin to AdoCbl.

Moreover, controls showed that the difference in CM-Cbl formation between the chemical and enzymic systems could not be accounted for on the basis of differences in the kinetics of cob(1)alamin formation. When DTT plus FAD were used to generate ‘free’ cob(1)alamin, the rate of cob(1)alamin production was similar to the rate observed for the PduS–PduO system. (In the absence of iodoacetate, assays that contained DTT, FAD and purified PduO produced AdoCbl at a rate of 3·5 nmol min⁻¹, which was very close to the rate at which AdoCbl was produced in assays containing the PduS and PduO enzymes, 3·8 nmol min⁻¹).

It is also of note that iodoacetate had relatively little effect on the total amount of AdoCbl produced from cob(II)alamin by the PduS and PduO enzymes (Table 1, rows 1 and 3). In the absence and presence of iodoacetate, 128 and 114 nmol AdoCbl was produced, respectively. This indicates that the majority of the cob(1)alamin formed [during the conversion of cob(II)alamin to AdoCbl by the PduS–PduO system] was sequestered.

**PduS does not produce significant amounts of cob(1)alamin in the absence of the PduO protein**

We also used iodoacetate to test whether the PduS protein can produce ‘free’ cob(1)alamin in the absence of the PduO protein. Assay mixtures were prepared that were similar in composition to the PduS–PduO linked assay described above except that PduO was omitted and iodoacetate was included. Iodoacetate reacts with cob(1)alamin to form CM-Cbl, with a concomitant increase in absorbance at 525 nm that provides a facile method for quantifying ‘free’ cob(1)alamin that is slightly more sensitive than the linked assay with the PduO adenosyltransferase. In assays containing iodoacetate, no cob(1)alamin was detected over a 30 min period (Fig. 5). In contrast, cob(1)alamin was formed at a rate of 3·9 nmol min⁻¹ in the linked cob(II)alamin reductase assays that included the PduO enzyme. These findings show that PduS enzyme requires the presence of the PduO adenosyltransferase in order to reduce cob(II)alamin to cob(1)alamin at a detectable rate, which indicates a physical interaction between the PduS and PduO enzymes.

Controls showed that AdoCbl was formed at a rate of 3·9 nmol min⁻¹ in linked cob(II)alamin reductase assays supplemented with iodoacetate, which demonstrated that iodoacetate was not inhibitory to the PduS enzyme. In addition, when 1 mM DTT and 50 μM FAD were used to generate cob(1)alamin chemically, CM-Cbl was formed at a rate of 38·2 nmol min⁻¹, showing that iodoacetate was an effective trap for cob(1)alamin under the assay conditions used. Lastly, controls also showed that neither heat-treated PduO nor BSA stimulated cob(1)alamin production by the PduS enzyme.

**DISCUSSION**

In this report, we showed that the PduS protein has both cob(III)alamin and cob(II)alamin reductase activity *in vitro*. The observed rates of cob(III)alamin (HOCbl) and cob(II)alamin reduction were 91 and 7·6 nmol min⁻¹ (mg crude PduS extract)⁻¹, respectively. Based on SDS-PAGE analyses, we estimate that the PduS extracts contained approximately 5% PduS protein (Fig. 3). Thus, it is expected that the specific activities of purified PduS would be roughly 1820 and 152 nmol min⁻¹ mg⁻¹ for HOCbl and cob(II)alamin reduction, respectively. It is likely that this level of activity would be adequate to meet physiological needs. Cells require AdoCbl in only very small quantities. Typical intracellular levels are about 1 μM, whereas other well-known coenzymes are present at 100- to 1000-fold higher concentrations or more (Schneider & Stroinski, 1987). Thus, the studies reported here indicate that the PduS enzyme is a bifunctional cobalamin reductase that has physiologically relevant levels of activity.
Of the enzymes that have been investigated, the corresponding gene is known in five cases. In *S. enterica*, the Fre protein was reported to catalyse the production of reduced flavin nucleotides which, in turn, reduce cob(II)alamin chemically to cob(III)alamin (Fonseca & Escalante-Semerena, 2000). The *E. coli* FldA protein, the human methionine synthase reductase (MSR) and the human novel reductase 1 (NR1) were shown to reduce cob(II)alamin to cob(I)alamin for the reductive activation of methionine synthase (Fujii *et al.*, 1977; Olteanu & Banerjee, 2003; Paine *et al.*, 2000; Wilson *et al.*, 1999). In addition, the FldA protein in combination with the Fpr protein was reported to reduce cob(III)alamin to cob(I)alamin for AdoCbl production by the CobA adenosyltransferase (Fonseca & Escalante-Semerena, 2001).

The PduS protein is unrelated in amino acid sequence to the Fre, Fpr, FldA, MSR and NR1 proteins. Hence, PduS represents a new class of enzyme with cobalamin reductase activity. Analyses using BLASTP software showed that GenBank currently contains 52 proteins related in sequence to PduS (Expect value \(7 \times 10^{-10}\) or lower) (Altschul *et al.*, 1990, 1997). It is likely that the PduS homologues found in *Salmonella*, *Lactobacillus*, *Listeria* and *Klebsiella* have conserved functions, since their encoding genes are located proximal to genes involved in AdoCbl-dependent 1,2-PD degradation (Bork *et al.*, 1998; Overbeek *et al.*, 1999). However, PduS also has homology to the RnfC subunit of NADH:ubiquinone oxidoreductase, and many of the PduS homologues currently in GenBank are arranged with genes predicted to encode this enzyme (*rnfABCDGE*). These enzymes probably have functions divergent from PduS, but an intriguing possibility is that they have roles in both electron transport and cobalamin reduction. Also of interest is the fact that PduS has significant similarity to a single human flavoprotein (GI:20149568; Expect value \(8 \times 10^{-6}\), but not to other human electron transport oxidoreductases. This finding could be helpful to studies of cobalamin reduction in humans, which has relevance to several disease states including hyperhomocysteinuria, methylmalonic aciduria, cancer and heart disease (Ames, 2001; Rosenblatt & Fenton, 1999).

In this report, we also conducted studies to determine whether cob(I)alamin was sequestered or released 'free' in solution during the conversion of cob(II)alamin to AdoCbl by an *in vitro* system that contained the PduS cobalamin reductase and the PduO adenosyltransferase. Experiments in which iodoacetate was used as a chemical trap indicated that cob(I)alamin was sequestered during the conversion of cob(II)alamin to AdoCbl by the PduS–PduO system. This is likely to be physiologically important. Cob(I)alamin is one of the strongest nucleophiles that exist in aqueous solution and an extremely strong reductant (\(E_{\text{m}} = -0.61\) V) (Banerjee *et al.*, 1990; Schrauzer *et al.*, 1968; Schrauzer & Deutsch, 1969). It rapidly reduces protons to H$_2$ gas at pH 7 and is instantaneously oxidized by air (Schneider, 1987a, b). Hence, sequestration of cob(I)alamin would prevent a futile cycle in which cob(II)alamin reduction is followed by the non-specific oxidation of cob(I)alamin due to its high reactivity. Thus, the finding that cob(I)alamin is sequestered suggests a specific and physiologically important interaction between the PduS and PduO enzymes.

Earlier studies have shown that the PduO adenosyltransferase is a component of the polyhedral bodies involved in 1,2-PD degradation by *S. enterica* (Havemann & Bobik, 2003). Results presented here indicate that PduS interacts physically with PduO; hence, the PduS protein should also be associated with these polyhedra. Recently, the polyhedral bodies of 1,2-PD degradation were purified and found to consist of at least 15 different polypeptides (Havemann & Bobik, 2003). PduS was not among the proteins identified, but it could have been missed if it was a minor component. Alternatively, PduS could associate with the outer surface of the polyhedral bodies, in which case it might have been displaced by the detergent treatment used for polyhedral body purification.

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**REFERENCES**


genes constitute an operon. prpBCDE encodes a member of the sigma-54 family of activators, and the transcribed units comprise the


