Membrane topology analysis of the *Escherichia coli* cytosine permease

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The *Escherichia coli* *codBA* operon encodes cytosine permease (CodB) and cytosine deaminase (CodA). CodB mediates uptake of exogenously supplied cytosine, and CodA catalyses the hydrolytic deamination of cytosine to uracil and ammonia. The hydropathic profile of CodB indicates that it is an integral cytoplasmic membrane protein possessing several transmembrane-spanning domains. The membrane topology of CodB was investigated by using gene fusions containing varying lengths of the amino-terminus of CodB fused to either alkaline phosphatase (AP) or β -galactosidase (BG). The AP activities expressed by the CodB–AP fusions are consistent with a topological model in which the amino- and the carboxy-termini of CodB are located in the cytoplasm, and in which CodB possesses 12 membrane-spanning segments. The enzyme activities of most of the CodB–BG fusions support the model. However, the results obtained with some of the CodB–BG fusions illustrate the limitations of using BG as a reporter protein in studies of membrane protein topology.

Keywords: cytosine permease, membrane protein topology, gene fusion, *phoA*, *lacZ*

INTRODUCTION

The only known route by which cytosine can be metabolized is via hydrolytic deamination, catalysed by cytosine deaminase (CodA), to yield uracil and ammonia. CodA is found in a number of bacteria and fungi (Esders & Lynn, 1985; Hoepflich *et al.*, 1974; Ipata *et al.*, 1971; Katsuragi *et al.*, 1986; Sakai *et al.*, 1975; West *et al.*, 1982), but is absent from mammalian cells (Koechlin *et al.*, 1966). The *codA* gene, encoding CodA, is the second gene in the *codBA* operon located close to the *lac* operon at 8 min on the *Escherichia coli* chromosome (De Haan *et al.*, 1972). The first gene in the operon, *codB*, encodes cytosine permease (CodB), a membrane protein responsible for transport of cytosine across the cytoplasmic membrane (Danielsen *et al.*, 1992). Mutants deficient in uptake of cytosine, i.e. *codB* mutants, have been selected as clones resistant to low concentrations of the toxic cytosine analogue 5-fluorocytosine (Lind *et al.*, 1973). Pyrimidine-requiring *codB* mutants cannot grow with low concentrations of cytosine as sole pyrimidine source. *E. coli codA*

codB⁺ mutants are capable of concentrating externally applied cytosine 200-fold, and this capacity is further increased 10-fold when *codB* is present in multicopies on a plasmid (Danielsen *et al.*, 1992).

The use of gene fusion techniques has expanded our understanding of the organization of proteins in the lipid bilayer of the bacterial cytoplasmic membrane (for recent reviews see Boyd, 1994; Hennessey & Broome-Smith, 1993; Traxler *et al.*, 1993). The first reporter protein used specifically for the study of membrane protein topology was the periplasmic alkaline phosphatase (AP) from *E. coli*, encoded by the *phoA* gene (Boyd *et al.*, 1987; Manoil & Beckwith, 1986; Manoil *et al.*, 1988, 1990). Other reporter proteins include TEM β -lactamase encoded by the *bla* gene (Broome-Smith *et al.*, 1990) and *E. coli* β -galactosidase (BG) encoded by *lacZ* (Froshauer *et al.*, 1988).

The important property of AP is that it is enzymically active only when it is translocated through the cytoplasmic membrane into the periplasmic space. If mutations or gene fusions cause AP to be retained in the cytoplasm it is enzymically inactive, probably because formation of the intrachain disulphide bonds, essential for enzymic activity, does not occur in this compartment (Derman *et al.*, 1993a). When AP is fused to a membrane protein, the topogenic information present in the domain

Abbreviations: AP, alkaline phosphatase; BG, β -galactosidase; CodA, cytosine deaminase; CodB, cytosine permease; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The SwissProt accession number for the sequence data reported in this paper is P25525.

to which the fusion is made determines the ultimate cellular location of AP (Manoil & Beckwith, 1986). Consequently, when AP is fused to a membrane protein it results in low enzymic activity if the fusion junction is in a cytoplasmic domain, and high activity if it is located in a periplasmic domain.

A reverse pattern of enzyme activities is observed when BG is used as a reporter protein (Froshauer *et al.*, 1988; Lee *et al.*, 1989). Fused to a protein export signal, such as a periplasmic domain of a cytoplasmic membrane protein, BG becomes embedded in an enzymically inactive form in the cytoplasmic membrane during the translocation process. However, if the fusion occurs to a cytoplasmic domain BG will be attached to the carboxy-terminal end of the preceding transmembrane segment, and will be facing the cytoplasm in an enzymically active form. Thus the combined use of AP and BG fusions represents an efficient system for analysis of membrane protein topology (Manoil, 1990).

In the present paper the membrane topology of CodB has been studied by gene fusions to both AP and BG.

METHODS

Materials and culture conditions. Luria broth (LB) and M63 minimal medium were used as growth media (Miller, 1972). With M63, glucose (2 mg ml⁻¹) was used as carbon source. When required, supplements were added at the following final concentrations: thiamin, 1 µg ml⁻¹; Casamino acids, 2 mg ml⁻¹; ampicillin, 30 µg ml⁻¹; chloramphenicol, 30 µg ml⁻¹. The chromogenic indicators for AP, 5-bromo-4-chloro-3-indolyl phosphate (XP) (Bachem), and for BG, X-Gal (Sigma), were used at a final concentration of 40 µg ml⁻¹. L-[³⁵S]Methionine (1000 Ci mmol⁻¹; 37 TBq mmol⁻¹) was obtained either from New England Nuclear or from Amersham. Rabbit anti-AP serum was prepared by C. Gardel, and rabbit anti-OmpA serum was a generous gift from C. Kumamoto.

Fig. 1. Maps of plasmids pSD201 and pSD202. Plasmids were constructed as described in Methods. pSD201 was used for construction of *codB-phoA* fusions by exonuclease III and pSD202 was used as a cloning host for PCR-amplified *codB* fragments.

Table 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype	Reference or source
Strain		
DHB4	F' <i>lac pro lacI^h</i> / Δ(<i>ara leu</i>)7697 <i>araD139</i> Δ(<i>lac</i>)X74 <i>galE galK rpsL phoR</i> Δ(<i>phoA</i>) <i>PvuII</i>	Boyd <i>et al.</i> (1987)
DH5α	<i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1</i> <i>gyrA</i> (Nal ^r) <i>relA1</i> Δ(<i>lacZYA-argF</i>) U169 <i>deoR</i> [φ80 <i>dlac</i> Δ(<i>lacZ</i>)M15]	Woodcock <i>et al.</i> (1989)
Plasmid		
pACYC184	Cm ^R Tc ^R	Chang & Cohen (1978)
pJEL247	R1 derivative carrying <i>lacZ</i>	Valentin-Hansen (1986)
pUI310	pUC19 derivative carrying <i>phoA</i>	Varga & Kaplan (1989)
pSD112	pBR322 derivative carrying the <i>E. coli codB-A</i> operon	Danielsen <i>et al.</i> (1992)
pSD200	pUC19 derivative carrying <i>codB-phoA</i> genes	This study
pSD201	pACYC derivative carrying <i>codB-phoA</i> genes	This study
pSD202	pACYC derivative carrying <i>phoA</i>	This study

Table 2. CodB-AP fusion primers

Sequence (5' → 3')*	Resulting construct†	Fusion no.‡
ataatggaTCCCCACCTTTTTGC	CodB _{com}	—
gcgggatcggTTTCCGCGCCGACT	CodB _{Lys-018}	1
gcgggatcggATCATGATAGCTAAGAC	CodB _{Asp-051}	2
gttgatcggTAGCAGTGAAGGCAG	CodB _{Leu-100}	3
tccggatcggTGCCTTACCCACCG	CodB _{Ala-123}	4
accggatcggCGAAATGCCAAAAAAGAC	CodB _{Ser-148}	5
gcgggatcggATCTAACGGTTGTGCG	CodB _{Asp-192}	7
agcgatcggTTTGCCATTGCGAC	CodB _{Lys-222}	8
gcaggatcggATCAGAGATATCCGC	CodB _{Asp-257}	13
gggttgatcggCATCCCGGTAATGTT	CodB _{Met-297}	15
caggatcggATTGTTATACAGCCATAA	CodB _{Asn-320}	16
aattggatcggCATACGCGTGGTCGC	CodB _{Met-358}	21
accggatcggTCCCGGTAACCACTG	CodB _{Gly-381}	22
gtaggatcggTTCGACACTGTTAGCCTCC	CodB _{Glu-419}	23

* Letters in uppercase represent *codB* residues. Underlined bases show the *Bam*HI linker introduced in all amplified fragments to facilitate the subsequent cloning; the last *codB* codon present in the amplified fragment is shown in bold next to the proline codon (italics) introduced to give all fusions the same linker region. Note that the sequence of all primers, except CodB_{com}, is complementary to the sense strand of *codB*.

† Last CodB residue present in the amplified fragment.

‡ Number of the resulting constructs refers to Table 3 and Fig. 3.

Recombinant DNA techniques and sequencing. DNA manipulations, transformations, restriction analysis and gel purification of DNA fragments were performed by standard procedures according to Sambrook *et al.* (1989). Enzymes for molecular cloning and restriction analysis were obtained from Promega or New England Biolabs. DNA sequence analysis was accomplished by the chain-termination method of Sanger *et al.* (1977) using double-stranded plasmid DNA template. Oligonucleotides used for sequencing of the *codB-phoA* and *codB-lacZ* fusion junctions were 5'-ATCACGCAGAGCGGCAGTCTGAT-3' (*phoA*) and 5'-CGCCAGGGTTTCCAGTCACGA-3' (*lacZ*).

Bacterial strains and vectors. Bacterial strains and plasmids are listed in Table 1. Plasmid pSD112, which was used for PCR amplification of *codB* fragments, is described elsewhere (Danielsen *et al.*, 1992). Plasmid pUI310 (Varga & Kaplan, 1989) is a pUC19 derivative that carries a multiple cloning site in front of the coding region for signal-less AP and was a gift. Plasmid pACYC184 was from New England Biolabs. Plasmid pJEL247 (Valentin-Hansen, 1986) is a single copy mini-derivative of R1, used in the construction of the *codB-lacZ* fusions and was a gift.

Plasmid constructions

pSD200. A 1.5 kbp fragment containing the *codB* gene was obtained by PCR amplification using pSD112 as template and the following primers: 5'-TTACACCGATAAGCTTTCCC-CC-3', which introduces a *Hind*III site in the 5' end of the amplified fragment, and 5'-GCCACAGCCCCTCTTCGCCTGG-3'. The amplified fragment was subsequently cloned as a *Hind*III/*Pst*I fragment into *Hind*III/*Pst*I-digested pUI310.

pSD201 (Fig. 1). The entire *codB-phoA* region of pSD200 was cloned as a 2.8 kbp *Hind*III/*Ban*II fragment into *Hind*III/*Ban*II-digested pACYC184.

pSD202 (Fig. 1). The *phoA* region of pUI310 was cloned as a *Hind*III/*Ban*II fragment into *Hind*III/*Ban*II-digested pACYC184.

Amplification of DNA. PCR (Saiki *et al.*, 1988) was performed on a DNA Thermal Cycler (Perkin Elmer) using *Vent* DNA polymerase (New England Biolabs). Reactions were done in a final volume of 100 µl using the buffer recommended by the supplier and 2.5 U DNA polymerase, 50 ng template, 100 pmol of each oligonucleotide primer and 25 nmol of each of the four dNTPs.

Enzyme assays on whole cells. Fresh stationary-phase cultures in LB were diluted 100-fold, grown for 3 h at 37 °C, harvested, and permeabilized by SDS/chloroform treatment (Miller, 1972). AP activity was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis (Manoil, 1991). To prevent spontaneous activation of low-activity AP fusions in non-growing cells 1 mM iodoacetamide was routinely added prior to assay (Derman *et al.*, 1993b). BG activity was assayed by measuring the rate of hydrolysis of *o*-nitrophenyl galactoside (Miller, 1972).

Construction of gene fusions by exonuclease III nested deletions. CsCl-purified plasmid pSD201 (Fig. 1) was digested with restriction endonucleases *Mlu*I and *Pst*I, phenol/chloroform extracted, ethanol precipitated and redissolved. Unidirectional deletions were generated by digestion with exonuclease III essentially as described by Sambrook *et al.* (1989). DNA was subsequently treated with mung bean nuclease and Klenow fragment to blunt-end the DNA, recircularized with T4 DNA ligase and transformed into DH5α. Transformants expressing high AP activity were selected as blue colonies on LB plates containing XP and chloramphenicol. Plasmid DNA from purified colonies was analysed by restriction endonuclease digestion and DNA sequencing.

Construction of gene fusions by use of PCR. DNA fragments including 85 bp of the region 5' of the *codB* promoter and various amounts of the *codB*-coding region were generated by PCR, using the primers shown in Table 2. All primers contained a *Bam*HI site at the 5' end. One primer, codB_{com}, contained

sequences corresponding to the region 85 bp upstream of the *codB* promoter, and was used together with each of the fusion-point-specific primers designed to be complementary to the sequence of the desired fusion-point. A proline codon (CCG) was introduced in all the latter primers to ensure that all fusions had identical linker regions, i.e. CodB-Pro-Asp-Pro-Arg-AP. Following amplification, the PCR products were digested with *Bam*HI and cloned into the pACYC184-derived *phoA* fusion vector pSD202 (Fig. 1). The resulting plasmids were transformed into DHB4 and Cm^R transformants isolated. The fusion junction of each plasmid was verified by DNA sequencing. The linker sequences were chosen for convenience. The inclusion of an arginine residue in the linker might, in some cases, result in poor export of AP fused to a periplasmic domain with a weak export signal because positively charged residues can act as determinants of cytoplasmic localization (Boyd & Beckwith, 1989, 1990). In one instance, inclusion of a single arginine residue in a linker sequence did not interfere with reporting of topology (Prinz & Beckwith, 1994).

The same PCR fragments used above for construction of the CodB-AP fusions were cloned as translational fusions with *lacZ* in the low-copy-number fusion vector pJEL247. The resulting CodB-LacZ fusion proteins all had the following linker region: CodB-Pro-Asp-Pro-Ser-Thr-Ser-Gln-Ala-Cys-Asp-Pro-LacZ. Plasmids were transformed into strain DH5 α , and AP^R transformants were isolated. The fusion junction of each plasmid was verified by DNA sequencing.

Rate of hybrid protein synthesis. Cells expressing the fusions were grown to mid-exponential phase at 37 °C in M63, supplemented with all common amino acids except methionine. At a cell density of approximately 10⁸ cells ml⁻¹ L-[³⁵S]methionine (30 μ Ci ml⁻¹; 1110 kBq ml⁻¹) was added to 1.5 ml of the culture. After 60 s, unlabelled methionine (100 μ g ml⁻¹) was added, and 750 μ l immediately withdrawn (pulse sample) for determination of the rate of protein synthesis as described by Manoil (1991). After 30 min a second sample (chase sample) was withdrawn for determination of the stability of the hybrid proteins. Immunoprecipitation was performed with antibodies to AP and OmpA (internal control) in the presence of the protease inhibitor PMSF (150 μ g ml⁻¹; Sigma). The precipitates were fractionated by SDS-PAGE (Laemmli, 1970), and the amount of radioactivity in the AP-specific and OmpA-specific polypeptides was quantified by a Phosphor-Imager. The amount of AP-specific radioactivity in the pulse samples, corrected for the number of methionine residues present in the fusion, was divided by that of OmpA and was used as a measure of the rate of synthesis of the fusion protein.

RESULTS

Prediction of the transmembrane topology of CodB

The alternating pattern of hydrophilic and hydrophobic segments seen in a hydropathy plot is a feature characteristic for membrane proteins with multiple membrane-spanning domains (Kyte & Doolittle, 1982). Positions of putative transmembrane-spanning segments in the amino acid sequence of CodB were identified using TopPred II (Claros & von Heijne, 1994), a computer program which generates the hydrophobic profile. Fig. 2 shows the hydropathy plot and indicates the position of 12 hydrophobic segments (I–XII) capable of spanning the cytoplasmic membrane. The prevalence of positively charged amino acid residues (arginine and lysine) in the first, third,

seventh, eleventh and thirteenth hydrophilic segments of CodB suggests, in accordance with the so-called positive inside rule (von Heijne, 1992), that these segments are cytoplasmic loops. By alternating the orientation of the 12 hydrophobic segments a topological model can be generated in which the amino- and carboxy-termini of CodB are located in the cytoplasm.

Construction of *codB-phoA* fusions

Random CodB-AP fusions were generated in plasmid pSD201 by unidirectional exonuclease III digestion into the 3' end of *codB* (Fig. 1, Methods). Following circularization the resulting plasmids were transformed into DHB4 and transformants expressing high levels of AP, due to in-frame fusions of AP to periplasmic domains of CodB, were isolated as blue colonies on plates containing chloramphenicol and XP. Cells harbouring fusions with low AP activity cannot be distinguished from cells containing no fusions or out-of-frame fusions. Fifty transformants expressing high levels of AP were isolated. Sequencing of plasmid DNA from these transformants identified 10 different CodB-AP fusion points (Table 3).

To obtain a more complete set of CodB-AP fusions PCR was used to construct fusions in which the fusion-point was located at the carboxy-terminus of each hydrophilic region throughout CodB (Boyd *et al.*, 1993). This will reduce the likelihood of isolating fusions expressing intermediate AP activities due to disruption of known topogenic determinants such as membrane-spanning segments or short hydrophilic segments with net positive charge (Boyd & Beckwith, 1990; Calamia & Manoil, 1990; Traxler *et al.*, 1993). Following this rationale a total of 13 plasmid-borne CodB-AP fusions were constructed (Table 3) and transformed into DHB4.

AP activities of CodB-AP fusions

AP activity was measured in DHB4 expressing the various CodB-AP fusion proteins and the relative rate of synthesis of each fusion protein was determined by pulse-labelling (see Methods). By dividing the steady-state AP activity with the rate of synthesis of the fusion protein a normalized AP activity was obtained which reflected the potential of each fusion for AP translocation. The results are summarized in Table 3, and the normalized AP activities of each fusion protein are shown in Fig. 3 superimposed on the 12-helical model for CodB. The observed enzyme activities correlate well with the proposed topological model; cells carrying fusions with the junction in, or near, the predicted periplasmic regions of CodB (hydrophilic loops 2, 4, 6, 8, 10 and 12) express AP activities 20–200 times higher than those in which fusion junctions were in predicted cytoplasmic regions (hydrophilic loops 1, 3, 5, 7, 9, 11 and 13). Accordingly, colonies expressing fusions to predicted periplasmic regions were blue on plates containing XP, whereas colonies expressing fusions to predicted cytoplasmic regions were white or pale blue.

Fig. 2. Hydropathy plot of CodB from *E. coli*. Hydropathy analysis was performed as described by Claros & von Heijne (1994), using a 19 amino acids moving window. The linear sequence of CodB is indicated below the hydropathy plot as a bar extending from the amino (N)- to the carboxy (C)-terminus. The hatched boxes within the bar as well as the roman numerals below the bar indicate positions of putative transmembrane segments.

Table 3. AP and BG activities

Fusion no.	Last CodB residue in fusion	Method of construction	Steady-state AP activity*	Relative CodB-AP synthesis rate†	Normalized AP activity‡	BG enzyme activity§
1	Lys-018	PCR	4.2	13	0.4	210
2	Asp-051	PCR	500	12	50	4.9
3	Leu-100	PCR	< 0.2	1.2	0.3	23
4	Ala-123	PCR	50	1.5	40	0.4
5	Ser-148	PCR	0.4	0.8	0.6	36
6	Asp-181	ExoIII	39	1.2	39	—
7	Asp-192	PCR	10	0.8	15	14
8	Lys-222	PCR	0.3	1.3	0.3	14
9	Leu-234	ExoIII	60	0.9	80	—
10	Ile-241	ExoIII	67	1	80	—
11	Gly-243	ExoIII	67	1	80	—
12	Gly-246	ExoIII	69	1.2	69	—
13	Asp-257	PCR	84	1	100	< 0.2
14	Gly-263	ExoIII	63	1	75	—
15	Met-297	PCR	0.9	0.8	1.4	51
16	Asn-320	PCR	63	0.8	94	0.4
17	Gly-323	ExoIII	69	1	82	—
18	Ser-329	ExoIII	52	1.1	59	—
19	Ala-330	ExoIII	47	0.8	70	—
20	Ile-332	ExoIII	43	0.8	63	—
21	Met-358	PCR	0.5	0.6	1	54
22	Gly-381	PCR	60	0.7	98	< 0.2
23	Glu-419	PCR	0.6	1.3	0.6	84

* Measured in permeabilized cells and given as nmol NPP hydrolysed min⁻¹ OD₆₀₀⁻¹. Values represent the mean of four to six independent determinations.

† Determined by pulse-labelling as described in Methods. Values are expressed relative to the rate determined for CodB₂₅₇-AP.

‡ Calculated by dividing the value for steady-state AP activity (column four) with the relative CodB-AP synthesis rate (column five), and expressed relative to the value for the CodB₂₅₇-AP fusion, set at 100.

§ Mean of four to six independent determinations, expressed in Miller units.

CodB-BG fusions

To investigate whether the use of BG as reporter protein would indicate a similar topological structure of CodB, the same PCR fragments which were used for the construction of AP fusions were cloned into the low-copy-number *lacZ* fusion vector pJEL247 (Valentin-Hansen, 1986). The resulting plasmids were transformed into DH5 α , and the level of BG activity expressed by the fusion proteins was measured. The results are shown in Table 3.

DISCUSSION

In the present study, the membrane topology of the *E. coli* CodB, a protein involved in the active transport of the pyrimidine base cytosine, has been examined. Based on a computer-generated hydropathic profile, which takes into account the distribution of positively charged amino acids, a topological working model was generated. To test the model a total of 23 different plasmid-borne *codB-phoA* fusions and 13 different *codB-lacZ* fusions were constructed, which contained various amounts of the amino-

Fig. 3. Topological model of CodB in the *E. coli* inner membrane, and positions of fusions to CodB. (a) Putative membrane-spanning segments are shown as cylinders. The height of the bars inside the cylinders indicates the relative hydrophobicity of the segment. Thick lines symbolize hydrophilic segments. +, Arginine or lysine residue. Thin lines indicate fusion points. The number and the normalized AP activity of the fusion is shown. For fusions 9–12 and 18–20 the mean AP values are given; the individual values are listed in Table 3. (b) Amino acid sequence of CodB. Arrows point to the position of individual fusions. The putative membrane-spanning segments (*mss*) are underlined and numbered.

terminus of CodB fused in-frame to AP or BG, respectively. Close correlation was found between the predictions made from the model and the observed AP activities of the CodB–AP fusions. When AP was fused to the hydrophilic domains 2, 4, 6, 8, 10 and 12, the resulting fusion proteins exhibited high enzymic activity. These domains were predicted to face the periplasmic space in the native protein, and the high levels of AP indicate that the preceding transmembrane segments promote efficient translocation of AP into the periplasmic space. AP fusions to the carboxy-terminal end of the hydrophilic domains 1, 3, 5, 7, 9, 11 and 13 result in hybrid proteins exhibiting considerably lower AP activity, suggesting that these domains in CodB are facing the cytoplasm. The alternative explanation for the low activity of the latter fusions, that they might be synthesized at lower levels than the high activity fusions, was ruled out by measuring the rate of synthesis by pulse-labelling (Table 3). With the exceptions

noted below, all fusions were synthesized at similar levels and the normalized activities do not suggest a different topology.

All CodB–AP fusion proteins were equally unstable and almost completely degraded after a 30 min chase (data not shown). A similar pattern of uniform instability is rarely observed with Tn*PhoA*-generated fusions to other membrane proteins. It could reflect the instability of CodB itself, or be caused by the linker sequences, i.e. Pro-Asp-Pro-Arg or Thr-Leu-Glu-Asp-Pro-Arg for the PCR- and exonuclease-III-derived fusions, respectively. Two patterns of degradation were observed. Hybrid proteins with the fusion junction in putative cytoplasmic domains (low-activity fusions) were completely degraded, presumably because AP moieties located in the cytoplasm lack functional conformation. In contrast, hybrid proteins with junctions in putative periplasmic domains (high

activity fusions) always yielded an AP-sized fragment that remained stable during the chase. This may be due to rapid translocation of the AP moiety of these latter fusions. Once exported, the AP part will be protease resistant, enzymically active and hence detectable by anti-AP immunoprecipitation (Traxler *et al.*, 1992).

Fusion of AP or BG to residues 18 and 51 of CodB resulted in unexpectedly high levels of enzyme activity (Table 3). The pulse-label experiment indicated that the rate of synthesis of CodB₀₁₈-AP and CodB₀₅₁-AP was significantly higher than that of all later fusions. A computer search of the *codB* sequence, using the program 'Terminator' (Brendel & Tifonov, 1984; Devereux *et al.*, 1984) identified a putative weak transcriptional terminator at a position corresponding to codons 76–91, which could explain the observed step-down in expression level. A step-down in the amount of fusion protein has also been observed with the *E. coli* MalF protein (Boyd *et al.*, 1987; Froshauer *et al.*, 1988). In that case the preceding protein in the same operon, MalE, is produced at much higher levels. The different rates of synthesis of early and late MalF-AP fusions may indicate that the signal responsible for the step-down is at least partially within the *malF* gene.

According to the model, residue 192 in CodB is located at the end of the third periplasmic domain. However, the normalized activity of CodB₁₉₂-AP (no. 7, Table 3) is only 15 units, i.e. approximately 20% of the activity observed for the other periplasmic domain hybrids. The assignment of this domain to the periplasm appears to be correct, since the enzyme level of cells expressing CodB₁₈₁-AP (fusion no. 6, Table 3) is almost as high as the other periplasmic domain hybrids. Several factors may contribute to the inefficiency by which the AP moiety of CodB₁₉₂-AP is translocated across the membrane. One is that the membrane-spanning segment preceding the fusion point of CodB₁₉₂-AP, is one of the least hydrophobic in the protein. Another may be the positive charges on Lys₁₈₃ and on the Arg residue of the linker. This is supported by the higher activity observed with fusion CodB₁₈₁-AP from which Lys₁₈₃ is absent. Finally the cytoplasmic domain immediately preceding the CodB₁₉₂-AP fusion protein contains no obvious topogenic determinant, such as positively charged residues. Thus, efficient translocation of this domain, in the wild-type protein, may require CodB sequences absent in the fusion protein, i.e. information present in the distal part of CodB. A somewhat similar observation was reported for the *E. coli* lactose permease where the ninth membrane-spanning segment was a less potent export signal than the other membrane-spanning segments, and it was proposed that coinserion of the ninth and tenth membrane-spanning segments was required for efficient translocation of the intervening periplasmic domain (Calamia & Manoil, 1990).

Conflicting results have been reported when BG has been used as reporter protein in studies of membrane protein topology (Froshauer *et al.*, 1988; Georgiou *et al.*, 1988; Gött & Boos, 1988). The topology for CodB was

investigated using a set of CodB-BG fusions, in which the ends of all hydrophilic CodB domains were fused to BG. With the exceptions noted below the BG level for these fusions supported the topological structure as predicted by the AP fusions. BG fused to the end of hydrophilic domains 1, 3, 5, 9, 11 and 13 resulted in hybrid proteins expressing high BG activity, whereas fusions to the end of hydrophilic domains 2, 4, 8, 10 and 12 resulted in hybrid proteins expressing low BG activity. The fusion point of CodB₁₉₂-BG is located at the end of the sixth hydrophilic domain, believed to be periplasmic, and should therefore express BG at low levels. The relatively high BG activity observed for this fusion is in accordance with the low activity of the homologous CodB₁₉₂-AP fusion, and supports the proposed poor export competence of the fifth membrane-spanning segment. At present there is no obvious explanation for the low activity observed for CodB₂₂₂-BG, in which BG is fused at the end of the seventh hydrophilic domain. If the topology analysis of CodB had been based entirely on the results obtained with BG as the reporter protein it would have been impossible to assign a cellular location to the sixth and seventh hydrophilic domains of CodB.

To our knowledge this is the first topological structure reported for a membrane protein involved in prokaryotic pyrimidine metabolism. Reports on the topology of other membrane proteins related to nucleotide metabolism comes from studies on the yeast uracil permease (Silve *et al.*, 1991).

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