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Point Mutations in a Conserved Region (TonB Box) of *Escherichia coli* Outer Membrane Protein BtuB Affect Vitamin B_{12} Transport

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Uptake of cobalamins and iron chelates in *Escherichia coli* K-12 is dependent on specific outer membrane transport proteins and the energy-coupling function provided by the TonB protein. The *btuB* product is the outer membrane receptor for cobalamins, bacteriophage BF23, and the E colicins. A short sequence near the amino terminus of mature BtuB, previously called the TonB box, is conserved in all *tonB*-dependent receptors and colicins and is the site of the *btuB451* mutation (Leu-8→Pro), which prevents energy-coupled cobalamin uptake. This phenotype is partially suppressed by certain mutations in *tonB*. To examine the role of individual amino acids in the TonB box of BtuB, more than 30 amino acid substitutions in residues 6 to 13 were generated by doped oligonucleotide-directed mutagenesis. Many of the mutations affecting each amino acid did not impair transport activity, although some substitutions reduced cobalamin uptake and the Leu-8→Pro and Val-10→Gly alleles were completely inactive. To test whether the *btuB451* mutation affects only cobalamin transport, a hybrid gene was constructed which encodes the signal sequence and first 39 residues of BtuB fused to the bulk of the ferrienterobactin receptor FepA (residues 26 to 723). This hybrid protein conferred all FepA functions but no BtuB functions. The presence of the *btuB451* mutation in this fusion gene eliminated all of its *tonB*-coupled reactions, showing that the TonB box of FepA could be replaced by that from BtuB. These results suggest that the TonB-box region of BtuB is involved in active transport in a manner dependent not on the identity of specific side chains but on the local secondary structure.

The outer membrane of *Escherichia coli* contains numerous proteins that allow the passage of nutrients (for a review, see reference 30). The nonspecific OmpF and OmpC porins, the anion-selective PhoE porin, and the substrate-specific LamB and Tsx proteins allow equilibration of appropriate substrates across the outer membrane. Other outer membrane transport proteins carry out high-affinity binding and energy-dependent uptake into the periplasmic space of specific substrates that are poorly permeable through the porin channels or are normally encountered at very low concentrations (29). The BtuB, FepA, FhuA, IutA, FecA, and FhuE proteins are required for the uptake and utilization of cobalamins, ferric enterochelin (enterobactin), ferrichromes, ferric aerobactin, ferric dicitrate, and coprogen-rhodotorulic acid, respectively (2, 3, 10, 11, 14, 27, 40). The genes encoding each of these proteins have been mapped, cloned, and sequenced (8, 17, 19, 22, 24, 32). These active transport proteins are called TonB-dependent receptors because the uptake of their substrates requires the products of *tonB* and *exbB* (1, 9, 16).

Braun and colleagues suggested that the *tonB* product couples metabolic energy to the outer membrane receptor proteins (6, 13, 15). In the absence of TonB, the receptors bind their substrates but do not carry out active transport or the energy-dependent steps of bacteriophage and colicin penetration (13). TonB function may affect the conformation of the receptor proteins, since the specific binding to BtuB or FhuA of phages BF23 or T5 is much less effectively inhibited by vitamin B_{12} or ferrichrome, respectively, in *tonB*$^+$ cells than in *tonB* mutants (15, 26). Reynolds et al. (34) concluded that vitamin B_{12} (cyanocobalamin; CN-Cbl) was actively transported across the outer membrane and accumulated in the periplasmic space in *btuC* mutants which lack the cytoplasmic membrane transport system. In contrast to the wild-type process, labeled CN-Cbl was readily released from BtuC$^-$ cells by chase with unlabeled substrate or disruption of the outer membrane with chelators, and it was not converted into other metabolic species.

The need for TonB to serve as an energy-coupling factor was inferred from observations that transport across the outer membrane is driven by the proton motive force, which cannot exist across this porin-rich membrane. In wild-type cells, CN-Cbl uptake is inhibited both by protonophores, which dissipate the proton motive force, and by arsenate, which depletes nucleoside triphosphate pools (5). Ferric enterochelin uptake displays a similar dependence on both the proton motive force and phosphate bond energy (33). However, the *tonB*-dependent CN-Cbl accumulation in the periplasm in *btuC* mutants was strongly inhibited by protonophores but not by arsenate (34; R. J. Kadner, unpublished data).

Although direct evidence for an interaction between BtuB and TonB is not available, cellular localization studies suggest that TonB is periplasmic, where it could make contact with the outer membrane receptors (31). Genetic studies suggest that sequences near the amino terminus of BtuB interact with TonB. An eight-amino-acid region, characterized by invariant Thr and Val residues and termed the TonB box (21), is strongly conserved among all TonB-dependent receptors and colicins (Table 1). The *btuB451* mutation, which converted Leu-8 in the middle of the TonB box to Pro, results in an energy-uncoupled phenotype (2,

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TABLE 1. Conserved sequences in TonB-dependent proteins: TonB box-I[a]

Protein	Residue	Sequence	Reference
BtuB	6	Asp **Thr** Leu Val **Val** Thr Ala Asn	17
FepA	12	Asp **Thr** Ile Val **Val** Thr Ala Ala	24
FhuA	7	Asp **Thr** Ile Thr **Val** Thr Ala Ala	8
IutA	6	Glu **Thr** Phe Val **Val** Ser Ala Asn	22
FecA	24	Phe **Thr** Leu Ser **Val** Asp Ala Ser	32
FhuE	6	Asp **Thr** Val Ile **Val** Glu Gly Ser	36
Cir	6	Glu **Thr** Met Val **Val** Thr Ala Ser	28
Colicin B	18	Asp **Thr** Met Val **Val** Trp Pro Ser	21
Colicin M	2	Glu **Thr** Leu Thr **Val** His Ala Pro	21

[a] TonB boxes are segments conserved among the TonB-dependent outer membrane transport proteins. The location of TonB box-I was defined by Lundrigan and Kadner (24). The invariant residues are in boldface type.

17). The mutant protein binds all its ligands normally, but the energy-dependent uptake of CN-Cbl is totally blocked. Heller et al. (18) isolated extragenic suppressor mutations that partly restored the defect in CN-Cbl utilization in a *btuB451* strain carrying the *tonB* gene on a multicopy plasmid. Many suppressor mutations were in the plasmid-borne *tonB* gene and converted Gln-165 to Leu or Lys, which suggested that a direct interaction occurs between TonB and the TonB box region of BtuB.

This report describes experiments that examine the role of amino acid residues in the TonB box region in transport activity of BtuB and of a hybrid BtuB-FepA protein. More than 30 amino acid substitutions within the eight-amino-acid TonB box were generated by doped oligonucleotide-directed mutagenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains used in this study are listed in Table 2. Plasmid pML261 carries a 2.4-kilobase (kb) *Cla*I fragment with the intact *btuB* gene in pUC8 (12). The *btuB451* mutation was introduced by replacing the 1.5-kb *Eco*RI fragment of pML261 with the corresponding fragment from a lambda phage carrying BtuA (isolated by Knut Heller), yielding pML262 (Fig. 1). This plasmid in a *btuB* host conferred susceptibility to phage BF23 and colicins E1 and E3 but did not allow CN-Cbl utilization. Plasmids carrying *btuB-fepA* hybrid genes were constructed by ligating the 3.5-kb *Nar*I-*Pst*I fragment from the *fepA*-containing plasmid pBB5 (24) into the 3.2-kb *Acc*I-*Pst*I fragments from pML261 and pML262, yielding pML263 and pML264, respectively. Plasmid pBJM2 is pACYC177 carrying the 1.7-kb *Hin*cII *tonB*$^+$ fragment and confers kanamycin resistance (26). Plasmid pAG1 was derived from pML261 by replacement of the *Hin*dIII sites in the multiple

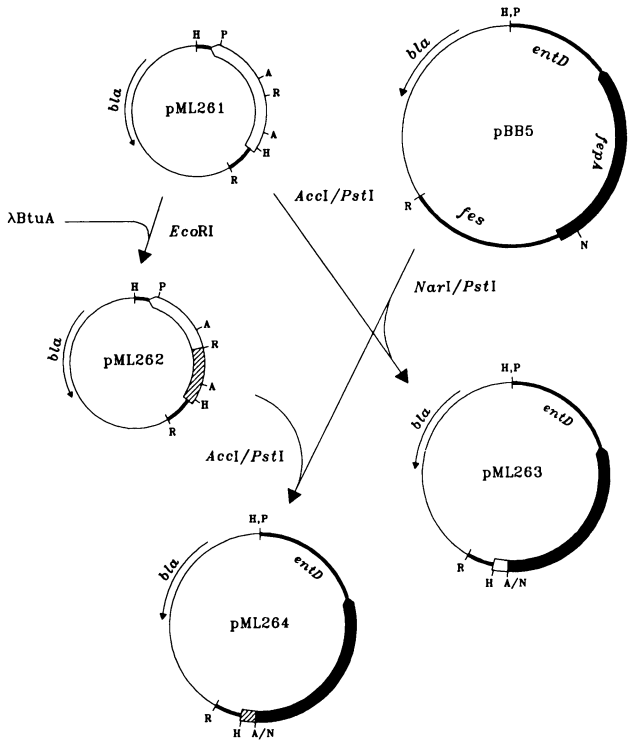

FIG. 1. Schematic diagram of construction of plasmids carrying *btuB-fepA* hybrid genes. Plasmids are drawn to scale. The thin lines represent vector pUC8 plasmid, with the small arrows indicating the *bla* gene. The thicker lines represent insert DNA, and the large arrows indicate the coding sequence for *btuB* (open arrows) and *fepA* (closed arrows). Hatched boxes represent areas derived from the *btuB451* allele. The restriction enzymes used to generate the fragments that were ligated together to form the product plasmids are listed beside each arrow. Abbreviations: A, *Acc*I; H, *Hin*dIII; N, *Nar*I; P, *Pst*I; R, *Eco*RI.

cloning region with an *Xho*I site (12). Derivatives of pAG1 carrying single-base substitutions and the mutations themselves are denoted by the resulting amino acid changes. For example, plasmid pLP8 encodes a BtuB protein with proline in place of leucine at position 8 of the mature sequence.

Genetic techniques. Standard methods were used for isolation of plasmid DNA, digestion with restriction endonucleases, separation by agarose gel electrophoresis, ligation, and transformation into competent cells (4, 25). Plasmid-bearing transformants were selected and maintained on media containing the appropriate antibiotic at 25 μg/ml. Susceptibility to colicins was determined by spotting serial dilutions of crude preparations of the colicins onto a lawn of the test strain in L broth-soft agar. Susceptibility is reported

TABLE 2. *E. coli* strains and characteristics

Strain	Genotype	Source or reference
CJ236	*dut-1 ung-1 thi-1 relA1*(pCJ105)	Bio-Rad
MV1190	Δ(*lac-proAB*) *thi supE44* Δ(*srl-recA*)*306*::Tn*10*(F′ *traD36 proAB lacI*^{q}ZΔ*M15*)	Bio-Rad
JM101	*thi supE44 relA1* Δ(*lac-pro*)(F′ *traD36 proAB*$^+$ *lacI*^{q}ZΔ*M15*)	25
RW193	*thi proC leu trpE lacY rpsL galK ara entA mtl xyl azi tsx supE44*	C. Earhart
UT6900	*thi proC leu trpE lacY rpsL galK ara purE mtl xyl azi tsx supE44 tonA* Δ*fepA*	27
RK4379	(*argF-lac*)*U169 araD139 relA1 rpsL150 flbB5301 deoC1 thi non gyrA219 metE70*	19
RK4795A	RK4379 Δ*btuB493 polA1 zig* Tn*10*	12
RK5016	RK4379 *argH1 recA56 btuB461*	19

TABLE 3. Growth and siderophore production on chrome azurol S plates and response to colicins D and B

Strain	Mean colony diameter (mm ± SEM)	Mean halo diameter (mm ± SEM)	$(A_H - A_C)/A_C$[a]	Titer	
				ColD	ColB
JM101 (wild type)	1.20 ± 0.07	2.62 ± 0.31	3.8 ± 0.8	10^5	
RW193 (*entA*)	0.96 ± 0.11	0.96 ± 0.11	0	$>10^8$	10^6
UT6900 (*fepA*)	0.56 ± 0.05	3.20 ± 0.07	32.5 ± 7.4	<1	10^2
UT6900(pML263)	0.94 ± 0.29	1.47 ± 0.72	0.6 ± 1.0	$>10^8$	10^6
UT6900(pML264)	0.52 ± 0.19	2.80 ± 0.57	34.8 ± 18.6	<1	10^2

[a] Values (mean plus or minus standard error of the mean) were determined as area of halo (A_H) minus area of colony (A_C) divided by area of colony.

as the highest dilution of colicin that gives a clear zone of killing.

Biochemical techniques. Rates of transport of CN[^{57}Co]Cbl were measured as previously described (34). Binding of cobalamin was measured with outer membranes prepared by the method of Schnaitman and MacDonald (37).

Production of enterochelin was estimated from the diameter of the orange zones surrounding individual bacterial colonies on chrome azurol S plates (39). Relative levels of enterochelin synthesis were compared by subtracting the area of each colony from the area of the surrounding halo and dividing by the area of the colony.

Oligonucleotide-directed mutagenesis. The technique of Kunkel et al. (23) was used for site-directed mutagenesis of the region encoding the TonB box. An M13mp18 derivative carrying the 1.8-kb *Hin*dIII-*Pst*I fragment of *btuB* was grown for two cycles in the *dut ung* strain CJ236. Several types of mutagenic 24-mer oligonucleotide were synthesized on an instrument from Applied Biosystems, Inc., Foster City, Calif., by using phosphoramidate chemistry. About 20 amino acid substitutions were obtained by use of a single oligonucleotide (5'-GATACTCTCGTCGTTACTGCTAAC) synthesized with 6% doping, i.e., in which each bottle of nucleotide precursors was contaminated to roughly 2% with each of the three wrong bases (20). Other mutations were obtained by using oligonucleotides with the same sequence as above but where the first two positions of a single codon (codon 7, 9, or 10) contained an equal mixture of all four bases. Finally, two mutations (LE8 and LQ8) were obtained by using oligonucleotides with the desired sequence.

Hybridization of the mutagenic oligonucleotides to single-stranded, uracil-containing viral DNA, synthesis of the complementary strand, and transfection into strain MV1190 were performed by procedures provided with the Mutagene kit (Bio-Rad Laboratories, Richmond, Calif.). Phage plaques were purified, and the nucleotide sequences of a portion of their inserts were determined by the dideoxy-chain termination technique, with universal primer (35).

Characterization of *btuB* mutants. Full-length *btuB* genes were constructed by inserting the 1.8-kb *Hin*dIII-*Pst*I fragment from the replicative form of M13mp18 derivatives with *btuB* point mutations into the appropriate *Hin*dIII-*Pst*I fragment of plasmid pAG1. Ligation products were introduced into strain RK5016, with selection for ampicillin resistance. The presence of the mutation in these plasmids was verified by cloning the *Hin*dIII-*Pst*I fragment from the plasmid back into M13mp18 and determining its nucleotide sequence.

The mutated *btuB* genes were transferred onto the chromosome in the haploid state by introducing each plasmid into the *polA* Δ*btuB493* strain RK4795A, as previously described (12). The *btuB493* mutation in this strain was generated by excision of a Tn*10* insertion in *btuB* and retains sufficient *btuB* sequence to allow plasmid integration by homologous recombination. The resulting strains were characterized for susceptibility to phage BF23 and for growth on minimal medium with 5 nM CN-Cbl in place of methionine.

Isolation of suppressors of mutations in the TonB box region. To isolate suppressor mutations that correct the Btu$^-$ phenotype of the LP8 and VG10 mutations, the corresponding plasmids were introduced into strain RK5016 along with the *tonB*$^+$ plasmid pBJM2. Btu$^+$ variants were selected on minimal medium with 5 nM CN-Cbl, ampicillin, and kanamycin; these arose at a frequency of around 10^{-7}. Plasmids were extracted from independent isolates and were introduced by transformation into RK5016(pBJM2), with selection on ampicillin and kanamycin. The Btu phenotype was determined on minimal medium with 5 nM CN-Cbl and by cross-streaking against BF23 or colicins E1 or E3. Transformation of plasmids from Btu$^+$ transformants was repeated into strain RK5016, with selection on ampicillin. Pseudorevertants in which the Btu$^+$ phenotype was associated with the BtuB plasmid were isolated, and the nucleotide sequence of the TonB box region was determined.

RESULTS

Properties of a BtuB-FepA hybrid protein. The Leu-8-to-Pro substitution in BtuB blocks TonB-dependent transport of vitamin B_{12} but has no effect on TonB-independent entry of phage BF23 or the E colicins. To determine whether this mutation would affect another *tonB*-dependent receptor, a hybrid gene was constructed in which the amino-terminal portion of BtuB was fused to most of the polypeptide chain of the TonB-dependent ferrienterochelin receptor protein, FepA (24). The promoter-proximal fragment of *btuB* generated by cleavage with *Acc*I (the *Sal*I site at nucleotide 485 [17]) was ligated to the promoter-distal fragment of *fepA* generated by cleavage with *Nar*I (at nucleotide 408 [24]). The resultant fusion gene encodes a hybrid protein that contains the signal sequence and first 39 residues of BtuB fused in frame to FepA sequences from the 26th residue to its C terminus.

Plasmid pML263 carrying the hybrid *btuB-fepA* gene was introduced into the *fepA* strain UT6900. The expected 81.5-kilodalton polypeptide, which was slightly larger than FepA (by 14 amino acids), was found in the outer membrane fraction. Synthesis of this protein was repressed by growth with 5 nM CN-Cbl, showing that its expression was controlled by the *btuB* regulatory region. The BtuB-FepA fusion protein appeared to carry out all of the TonB-dependent functions of intact FepA. The *fepA* host strain is defective in high-affinity iron uptake and, thus, exhibits increased production of enterochelin. Growth yield on iron-limiting medium and the extent of enterochelin production were estimated from the sizes of the bacterial colonies and the surrounding orange halos on chrome azurol S indicator plates (39) (Table 3). Enterochelin synthesis is repressed by intracellular iron, and strains lacking high-affinity iron up-

TABLE 4. Vitamin B_{12} transport activity of *btuB* missense mutations in the TonB box region

Amino acid substitutions at residue no.[a]:								Presence (+) or absence (−) of cobalamin utilization[b]	Cobalamin uptake[c]
6	7	8	9	10	11	12	13		
GAT	ACT	CTC	GTC	GTT	ACT	GCT	AAC		
Asp	Thr	Leu	Val	Val	Thr	Ala	Asn	+	10.4
Glu								+	9.8
Gly								+	10.4
	Ala							+	7.8
	Asn							+	6.6
	Leu							+	9.1
	Pro							+	11.5
	Ser							+	13.2
		Arg						+	6.4
		Gln						+	10.2
		Glu						+	11.0
		Ile						+	12.7
		Pro						−	<0.05
			Ala					+	15.0
			Cys					+	5.6
			Gly					+/−	2.4
			Ile					+	8.4
			Leu					+	8.5
			Pro					+/−	3.1
			Thr					+	10.8
				Ala				+	9.5
				Gly				−	0.05
				Ile				+	10.2
				Pro				−	ND[d]
				Asp				+	11.8
					Asn			+	5.0
					Ile			+	10.6
						Asp		+	9.3
						Gly		+	9.1
						Thr		+	10.5
						Val		+	10.8
							Thr	+	5.7
							Tyr	+	8.5

[a] The wild-type sequence is given along the top, and the amino acid substitutions resulting from each *btuB* point mutation are indicated underneath the residue affected.

[b] Presence or absence of growth on 5 nM CN-Cbl of strain RK5016 carrying plasmid pAG1 derivatives with the indicated *btuB* mutation is indicated.

[c] Values indicate the rate of CN-Cbl transport of strain RK4795A carrying the indicated point mutations in single copy. Uptake is expressed in picomoles per 10^9 cells in 50 min.

[d] ND, Not determined.

take secrete substantially higher amounts of enterochelin than do uptake-proficient strains (7, 39). After 24 h of growth, colonies of wild-type strain JM101 and *entA* strain RW193 were 1.20 and 0.96 mm in diameter and were surrounded by small or no halos, respectively. The *fepA* strain UT6900 formed smaller colonies (0.56 mm), with much larger halos than those around colonies of the other strains. The specific area of the orange halo (area of halo beyond each colony divided by area of colony) around UT6900 was about eight times larger than that around JM101. Transformants of UT6900 carrying pML263 gave colonies of wild-type size and greatly reduced enterochelin production, with a specific halo area about 20% that in JM101. Plasmid pML263 did not enhance growth or decrease siderophore production in a *tonB* mutant host.

Whereas the *fepA* strain UT6900 was fully resistant to colicin D, transformants carrying plasmids pBB5 (*fepA*$^+$) or pML263 (*btuB-fepA*) were as susceptible to the colicin as was strain RW193 (Table 3). Similar results were seen with colicin B. Thus, the BtuB-FepA hybrid protein encoded by pML263 appeared to carry out all of the TonB-dependent reactions of the intact FepA protein.

***btuB451* mutation in the BtuB-FepA chimera.** The analogous *btuB-fepA* hybrid gene was constructed by using the *btuB451* allele as the source of the promoter-proximal portion. When the resultant plasmid pML264 was introduced into strain UT6900, the fusion protein was found in the outer membrane fraction in amounts similar to that of the BtuB-FepA protein, and its synthesis was repressed by CN-Cbl. Despite the presence of the receptor protein, these cells displayed no TonB-dependent FepA functions, i.e., they grew poorly on iron-limited medium, secreted large amounts

of enterochelin, and were tolerant to colicins B and D (Table 3). Thus, the LP8 substitution impairs energy coupling not only in BtuB but also when the bulk of the polypeptide is from FepA. This result suggests that the amino terminus is directly involved in the TonB-dependent energy-coupling process.

Mutagenesis of the region encoding the TonB box. Oligonucleotide-directed mutagenesis allowed examination of the role of individual amino acid residues in the TonB box of BtuB in energy coupling. Several types of mutagenic oligonucleotide primers were prepared. Most of the mutants were obtained by using a single oligonucleotide in which roughly 2% of each of the wrong bases was incorporated at each position. In one experiment in which 210 plaques arising after complementary strand synthesis were sequenced, 40 (19%) carried a single base change within the mutagenized region. There was one case with two base changes. Altogether, there were 25 different base substitutions resulting in 19 codon changes. Each codon was converted into at least two other codons. Of the 24 nucleotides within the mutagenized region, changes were recovered in all but six. Transitions and transversions were obtained with equal probability, and base changes were substantially more likely when the parental nucleotide was a pyrimidine. Thus, the doped oligonucleotide procedure yielded a fairly random distribution of single mutations at reasonable frequency.

Two mutations within the TonB-box-coding region were obtained by using mutagenic oligonucleotides to convert the CTC codon for Leu-8 into CAG (Gln) or GAA (Glu). Other mutations were generated by oligonucleotide primers synthesized with an equal proportion of all four bases at the first two positions of an individual codon.

By these procedures, 32 amino acid substitutions were recovered within the eight-codon TonB box, including 25 of the 50 possible single-base changes that introduce a different amino acid (Table 4). To regenerate the full-length *btuB* gene, the 1.8-kb *Hin*dIII-*Pst*I fragments containing each mutation were cloned into the 3.3-kb *Hin*dIII-*Pst*I fragment from plasmid pAG1. The mutations are designated in terms of the original and the new amino acid inserted at a particular codon of the mature sequence; for example, the *btuB451* mutation is described as LP8. Each mutation was crossed onto the chromosome of the *polA* strain RK4795A to test its phenotype in single copy number.

Response to phage BF23 and E colicins. The response to phage BF23 and colicins E1 and E3 was determined for host strain RK5016 carrying each *btuB* point mutation on a multicopy plasmid and strain RK4795A with the same mutation on the chromosome. The host strains were completely resistant to phage BF23 and colicin E3 and were killed by colicin E1 only at a 10^5-times higher titer than was required for killing of the wild-type strain. In every case, strains carrying the TonB box mutations were as susceptible to these lethal agents as was the wild-type strain. These results indicated that the mutations did not significantly impair the synthesis of the receptor, its insertion into the outer membrane, or its substrate-binding properties.

Utilization of vitamin B_{12}. Growth of *metE* strains with 0.5 and 5 nM CN-Cbl in place of methionine provided an initial screen of TonB-dependent receptor function. Wild-type strains grow well on these concentrations, whereas *btuB*, *tonB*, or *btuB451* strains require 5 μM CN-Cbl (2). Of the 32 *btuB* point mutations affecting the TonB box, most conferred a wild-type growth response to CN-Cbl, whether they were carried on the plasmid or chromosome. The LP8 mutant isolated during this study was unable to grow on 5 nM CN-Cbl, as was the original *btuB451* strain. The VG10 and VP10 mutants were also completely negative, although the response of VP10 has only been tested on a plasmid. VG9 and VP9 showed reduced growth in this assay. All strains grew well with 5 μM CN-Cbl.

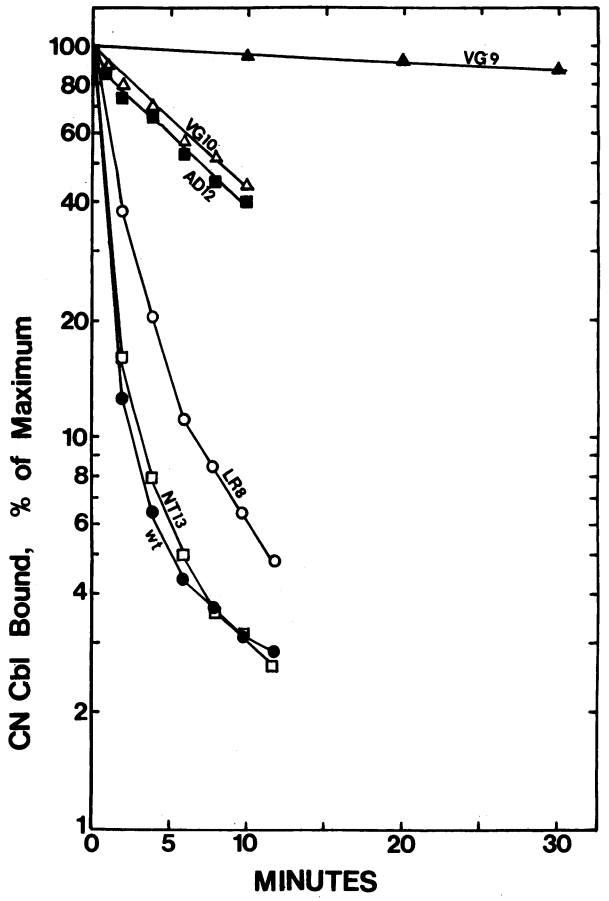

FIG. 2. Rates of dissociation of the CN-Cbl–BtuB complex in outer membrane particles. Each 2-ml reaction mixture sample contained 28 to 34 ng of outer membrane protein (prepared as described elsewhere [37]), 40 nM CN[^{57}Co]Cbl, and 100 mM potassium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 6.6. Incubations were done on a shaker at 37°C, and 200-μl samples were taken periodically. Cobalamin binding was measured by Millipore filtration and liquid scintillation counting (34). After 30 min of incubation, 5 μl of 5 mM nonradioactive CN-Cbl was added and further samples were taken at timed intervals. The results are expressed as percentages of the amount of label bound before addition of the chase solution. The outer membranes were from strains carrying *btuB* plasmids with the following variants of BtuB: wild type (wt), ●; NT13, □; LR8, ○; AD12, ■; VG10, △; and VG9, ▲. In each preparation, the BtuB protein was present at about 100× the normal haploid amount.

Vitamin B_{12} transport. The rate of uptake of CN[^{57}Co]Cbl uptake was measured in derivatives of strain RK4795A carrying the *btuB* point mutations in single copy (Table 4). Uptake activity in most strains was essentially identical to that in the strain with the *btuB*$^+$ allele. As expected from the growth results, the LP8 and VG10 substitutions eliminated energy-dependent accumulation of CN-Cbl. Uptake in the VG9 and VP9 mutants was reduced to about 20% of wild type, and the LR8, TN11, and NT13 mutants displayed about 50% of the wild-type rate of uptake. However, no single amino acid residue was essential for transport activity.

The properties of CN-Cbl binding to mutant BtuB proteins were determined with whole cells and isolated outer membranes from strains carrying plasmid pAG1 derivatives with selected point mutations (LR8, VG9, VG10, AD12, and NT13 [data not shown]). All strains possessed similar total levels of specific CN-Cbl binding (1.2 to 2.0 nmol/mg of protein), with similar affinities (K_d, 2 to 7 nM). These results confirmed that the point mutations in the TonB box did not markedly affect the amount of BtuB protein in the outer membrane or its affinity for substrate. However, substantial differences were seen in the rate of release of labeled CN-Cbl upon addition of excess nonradioactive substrate (Fig. 2). Membranes from the wild type and the NT13 mutant showed rapid release of label ($t_{1/2}$, <1 min), while release from LR8 was slightly slower ($t_{1/2}$, 1.5 min). In contrast, mutant alleles VG10 and AD12 showed a significantly slower off rate ($t_{1/2}$, 6.5 to 8.5 min), and the VG9 receptor gave extremely slow release ($t_{1/2}$, 150 min). As had been previously described for the LP8 mutant (34), alterations in the TonB box seem to affect the kinetics of substrate binding or release, possibly reflecting changes in the ability of the transport protein to undergo the reorientations necessary for energy-coupled substrate release into the periplasmic space.

Intragenic suppressors of TonB box mutations. Pseudorevertants carrying mutations in *btuB* that corrected the defect in CN-Cbl utilization of LP8 and VG10 were isolated. The nucleotide sequence in the TonB box region was determined to provide further information about the requirement for specific residues in this portion of the protein. Of 16 independent Btu$^+$ revertants of LP8, seven carried the wild-type sequence (CCC→CTC) and six replaced Pro-8 with Ser (CCC→TCC). In two revertants, the proline codon was still present at position 8 but Ala-12 was changed to Val (GCT→GTT). In another revertant which retained proline at residue 8, the two codons immediately preceding Ala-12 (GTTACT) were duplicated so that the 12th residue was now a valine. Thus, BtuB with proline at position 8 can function for transport if a valine is present four residues away.

A similar situation was seen in 14 independent Btu$^+$ revertants of VG10. In three isolates, Gly-10 was replaced with Ser (GGT→AGT). Eleven revertants of VG10 retained Gly-10 but had an isoleucine in place of Thr-7 (ACT→ATT). No revertants were found in which the TonB box region was not changed. A representative of each of these back mutations or intragenic pseudorevertants was crossed onto the chromosome. All showed the same growth response as the wild type at low CN-Cbl concentrations. Thus, even the amino acid substitutions which resulted in complete loss of transport activity could be tolerated when combined with other amino acid substitutions in the TonB box region.

DISCUSSION

The TonB-dependent outer membrane transport proteins appear to carry out the active transport of their substrates into the periplasmic space. The requirement for the TonB protein and the proton motive force suggests that these receptors may employ a novel mechanism of energy coupling, one that involves direct interaction of the TonB protein with specific domains on the receptor proteins. Sequences near the amino termini of the receptors have been suggested to play a central role in TonB-dependent energy coupling. This TonB box is highly conserved in all TonB-dependent proteins (28). Although it may lie a variable distance from the amino terminus, its consensus sequence is acidic-Thr-hydrophobic-Val/Ile/Thr-Val-polar-Ala-Ser/Ala/Asn. The LP8 change in BtuB resulted in an energy-uncoupled phenotype that was also observed for FepA ferrienterochelin receptor function when present as a BtuB-FepA hybrid protein.

The present study was undertaken to examine the role of the amino acids in the TonB box in transport function. A substantial number of mutant alleles was generated by doped oligonucleotide-directed mutagenesis. Even without any selection, single-base substitutions were recovered in about 20% of randomly picked plaques. More efficient recovery of mutations could be achieved by screening phage plaques by hybridization with the wild-type oligonucleotide under stringent conditions. Not all of the 152 possible amino acid substitutions were obtained, but half of the possible changes that would be generated by single-base change were recovered.

Despite the incomplete collection of amino acid substitutions, it was possible to draw several conclusions from the transport properties of the 32 *btuB* mutant alleles. Most of the mutations altering each position within the TonB box of BtuB had little effect on transport activity, even though the new amino acids were of a different chemical nature than the original. For example, Leu-8 could be replaced by isoleucine, glutamate, or glutamine with no change in transport. However, the TonB box plays an important role in active transport, as shown by the fact that several mutations (LP8, VG10, and VP10) conferred a completely uncoupled phenotype and the TN7, LR8, VG9, VP9, TN11, and NT13 mutants showed significantly decreased transport activity. The invariant Thr-11 could be changed to isoleucine without effect, although change to the polar asparagine impaired transport function. Surprisingly, the strongly conserved Ala-12 could be changed to threonine, glycine, aspartate, and valine without impairing transport activity, although AD12 did display a slower rate of substrate release. Thus, the presence of a specific side chain was not required at any position in the TonB box region for normal energy-coupled transport activity. Schöffler and Braun (38) have recently shown that the majority of amino acid substitutions within the TonB box of FhuA had little or no effect on transport function. The IP9 mutation, analogous to LP8 in BtuB, conferred greatly reduced activity. Interestingly, the VD11 form of FhuA was completely inactive, whereas the corresponding VD10 of BtuB displayed wild-type behavior.

Earlier studies on the rates of dissociation of the CN[^{57}Co]Cbl–BtuB complex showed that Cbl release from whole cells ($t_{1/2}$, ca. 5 min) was much slower than from isolated outer membrane particles ($t_{1/2}$, ca. 50 s), which suggested that the occupied substrate-binding site might not be exposed on the outer surface of the cell (34). Cobalamin release from whole cells of the transport-deficient, TonB box mutant LP8 was even slower ($t_{1/2}$, ca. 20 min), which was interpreted as another expression of its impaired transport activity. However, with outer membrane particles, in which the CN-Cbl presumably has access to both sides of the outer membrane, the rate of release in LP8 was identical to that in the wild type, which was consistent with other evidence that the affinity of BtuB for Cbl was the same in these two strains. Some of the new TonB box mutants showed slow release from both whole cells and outer membrane preparations (Fig. 2). The retention of the slow Cbl release by the outer membrane particles may have resulted from the use of a simplified procedure for preparing outer membranes that did not include exposure to EDTA or detergents (37). The correlation between slower off rates and impaired transport was not absolute. Mutant VG9 showed extremely slow

release but retained partial transport activity, while mutant AD12 showed significantly slowed rates of Cbl release from the outer membrane but normal rates of transport.

The mutations that strongly decreased transport activity changed residues 8, 9, or 10 to proline or glycine, the two amino acids most likely to disrupt local secondary structure. Thus, the TonB box region probably contributes to transport function by interaction with TonB protein or in another step of energy coupling in a manner dependent on its secondary or higher-order structure but not on sequence-specific interactions.

The amino acid substitutions that restored transport activity to the LP8 and VG10 mutants were consistent with this model. These changes were only found in the TonB box, although additional changes elsewhere in the gene would not have been detected. In the case of LP8, restoration of transport activity occurred upon conversion by a single transition event of Pro-8 to another residue (Leu or Ser) or upon replacement of Ala-12 with valine. Revertants of VG10 arose either by replacement of the Gly-10 or by changing Thr-7 to Ile. In addition to these results, the partial decreases in transport activity in LR8, TN11, and NT13 mutants may be related to effects on local secondary structure, but prediction of the structural consequences of these changes is not obvious.

Given the low degree of structural discrimination in the TonB box, it is probable that other portions of BtuB are also involved in interaction with TonB and participation in the energy-coupling process. The other conserved regions within the amino-terminal third of the TonB-dependent transporters are likely candidates, and experiments are in progress to examine their role in energy coupling. In other preliminary studies of extragenic suppression, we found that the LP8 and VG10 mutations can be suppressed by *tonB* mutations, although this response was tested only in strains with multiple copies of both genes. Quantitative measurements of the effect of suppressor mutations in *tonB* on transport activity in various *btuB* mutants may provide insight into the interactions of these two proteins.

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