JOURNAL OF BACTERIOLOGY, June 1977, p. 1292-1302

Vol. 130, No. 3
Printed in U.S.A.

Basis for the Observed Fluctuation of Carboxypeptidase II Activity During the Cell Cycle in BUG 6, a Temperature-Sensitive Division Mutant of *Escherichia coli*

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Received for publication 23 December 1976

Diaminopimelyl-D-alanyl carboxypeptidase (carboxypeptidase II) is most active at the time of division, whether measured in toluene-treated cells of *Escherichia coli* K-12 strain D11-1, fractionated by size, or in toluene-treated cells of the temperature-sensitive division mutant, BUG 6 (B. D. Beck and J. T. Park, 1976). The present investigation has now shown that, under conditions that permit division, the increased carboxypeptidase II activity in toluene-treated cells of BUG 6 is probably not due to protein synthesis. Although dividing cells are more permeable than nondividing cells, permeability differences are not sufficient to account for the changes in carboxypeptidase II activity. Thus, in the toluene-treated nondividing cells, carboxypeptidase II is present, but its activity is masked, which suggests the presence of an inhibitor. Another striking difference between nondividing and dividing cells is that carboxypeptidase II is much more readily released from dividing cells by both tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid and toluene treatment. Carboxypeptidase II was partially purified and found to be an 86,000-molecular-weight protein consisting of two 43,000-molecular-weight polypeptides. Tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid treatment of nondividing cells releases less than 10% of the carboxypeptidase II and other periplasmic proteins that are releasable from dividing cells.

During cell division in *Escherichia coli*, invagination of the cytoplasmic membrane and the rigid murein layer occurs, forming a septum perpendicular to the surface of the cell (6, 7). This process is rapidly followed by the ingrowth of the outer membrane and separation into two daughter cells. The mechanism by which septum formation and cell separation are regulated is unknown, but murein hydrolases have often been postulated to play a role in these processes (8, 11, 13, 26, 33). (In this paper, the term murein hydrolase refers to any enzyme that degrades either murein or a murein precursor at specific sites, not necessarily resulting in solubilization of murein.) A murein hydrolase that is relevant to division should be most active about the time of septum formation. We have shown that carboxypeptidase II, which cleaves the bond between diaminopimelic acid and D-alanine in the peptide side chain of murein (15, 22), is most active at the time of cell division as judged by its activity in cells of D11-1 sized by sucrose gradient centrifugation and rendered permeable by toluene (4). Furthermore, there was a transient 10-fold increase in the specific activity of carboxypeptidase II in BUG 6, a temperature-sensitive division mutant (24), when shifted from nondividing to dividing conditions (4). These observations led us to investigate further the division-associated increase in carboxypeptidase II activity. Several possible mechanisms for the change in activity were: (i) an increase in the total amount of carboxypeptidase II protein; (ii) activation or release from inhibition of the enzyme; and (iii) enhanced uptake or availability of the substrate, uridine diphosphate-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*m*-diaminopimelyl-D-alanine (UDP-MurNAc-tetrapeptide). In this paper we present a study of the factors affecting carboxypeptidase II activity in BUG 6 under dividing and nondividing conditions. This is an easily manipulated model system for cell division that exhibits a dramatic fluctuation in carboxypeptidase II activity during the cell cycle.

MATERIALS AND METHODS

Strains. The source and markers of BUG 6, D11, and D11-1 have been previously described (4). AB1157, F⁻ *thr leu thi lacY galK ara xyl mtl proA his*

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argE str tsx sup-37 (3), was obtained from Lars Burman. AX655, which is AB1157 with the Ts_{21-58} marker (2), and $P_{4\times8}$ Ts_{84}, Hfr, *leu* (28), were obtained from James R. Walker.

Growth conditions. The growth conditions have been described previously (4). For preparation of nondividing and dividing cells, cells were grown in E medium plus 0.5% glucose at 32°C for at least 120 min (to 0.3 units of absorbancy at 585 nm), shifted to 42°C for 60 min (nondividing conditions), and then shifted back to 32°C for 15 min (dividing conditions).

Enzyme assays. The assays for carboxypeptidase I, carboxypeptidase II, amidase, and penicillinase have been described earlier (4).

D-Lactate dehydrogenase (LDH) was assayed by following the oxidation of dihydronicotinamide adenine dinucleotide (Sigma) at 340 nm (30) with a Zeiss PMQ II spectrophotometer. The reaction mixture contained 10 to 50 μg of protein from crude cell extracts and was incubated for 10 to 30 min at 22°C. One unit of LDH is that amount of enzyme that will oxidize 1 μmol of reduced nicotinamide adenine dinucleotide in 60 min at 22°C.

Glucose-6-phosphate dehydrogenase (GDH) was assayed by following the reduction of nicotinamide adenine dinucleotide (Sigma) at 340 nm (20) with a Zeiss PMQ II spectrophotometer. The reaction mixture contained 30 to 150 μg of protein from crude cell extracts and was incubated for 60 min at 22°C. One unit of GDH is that amount of enzyme that will reduce 1 μmol of nicotinamide adenine dinucleotide in 60 min at 22°C.

Specific activity. The specific activity of the enzymes studied is expressed as the number of units per milligram of protein. Protein concentration was determined by the Lowry method (19).

Methods of enzyme preparation. The methods for toluene treatment and disruption by ultrasonic oscillation have been previously described (4). The tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) treatment as described by Levy and Leive (18) was performed with modifications (4).

Purification of carboxypeptidase II. Tris-EDTA extract was prepared from 90 liters of BUG 6 grown in E medium plus glucose at 32°C to 0.5 to 0.7 units of absorbancy at 585 nm. The extract was dialyzed for 40 h against 0.05 M β-mercaptoethanol, applied to a column (3 by 12 cm) of Whatman microgranular diethylaminoethyl (DEAE)-cellulose (DE52) donated by Barbara Young, and eluted with a 1.5-liter gradient of 2 to 100 mM potassium phosphate (pH 7.2) applied with a Buchler pump. Fifteen-milliliter fractions were collected at the rate of 340 ml/h with a Buchler Fractomette. Carboxypeptidase II eluted at 60 mM potassium phosphate in a total volume of 230 ml. Half of this enzyme was dialyzed overnight against β-mercaptoethanol and applied to a DEAE column (1.5 by 4.4 cm) to further concentrate the enzyme. A 60-ml gradient of 2 to 100 mM potassium phosphate (pH 7.2) was applied to the column, and 2-ml fractions were collected at the rate of 200 ml/h. The enzyme eluted at 43 mM potassium phosphate in a total volume of 26 ml. A 5-ml portion of this concentrated enzyme was applied to a column (25 by 50 cm) of Sephadex G-200 (Pharmacia). One-milliliter fractions were collected at the rate of 6 ml/h. The peak fractions from the Sephadex column, containing 77% of the total enzyme recovered, were then pooled.

SDS polyacrylamide gel electrophoresis. The sodium dodecyl sulfate (SDS) polyacrylamide gel system was that of Laemmli (17) using a slab gel apparatus as described by Reid and Bielski (25). Acrylamide and *N,N,N',N''*-tetramethylethylenediamine were purchased from Bio-Rad. SDS (Sigma), recrystallized according to the method of Platt (23), was donated by Suzy Torti.

Samples for gel electrophoresis were first dialyzed against 100 volumes of 4 mM dithiothreitol (Calbiochem) in dialysis tubing (Spectrapor I) that had previously been boiled in 1 M Na_2CO_3 for 120 min, rinsed in water, boiled in 1% Siliclad (Sargent-Welch) for 15 min, and rinsed in water (Barbara Young, personal communication). Samples were then lyophilized on a VirTis lyophilizer and taken up in water at a concentration of 1 to 2.5 mg of protein per ml.

After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue (Schwarz/Mann) in 10% acetic acid and 50% methanol for 60 min (32), with 0.05% $CuSO_4$, 0.05% Coomassie brilliant blue in 10% acetic acid, and then 50% methanol for 60 min (Gail Sonenshein, personal communication). Gels were destained in 30% methanol and 7.5% acetic acid for 120 min (Dana Boyd, personal communication) and in 5% methanol and 7.5% acetic acid for at least 16 h and then dried using a polyethylene drying apparatus (10).

RESULTS

Effect of chloramphenicol on cell division and carboxypeptidase II activity in BUG 6. Since BUG 6 divides in the presence of chloramphenicol after a shift from 42 to 32°C (24), we investigated the changes in carboxypeptidase II activity under these conditions. If the chloramphenicol-resistant division is to occur, the cells must be rapidly cooled to 32°C by swirling the culture flask in an ice bath. The need for rapid chilling is presumably due to the short half-life of the temperature-sensitive component at 42°C (24). After 5 min of chloramphenicol treatment, the incorporation of radioactive arginine into trichloroacetic acid-precipitable counts was 2% that of the untreated cultures.

After a shift from 42 to 32°C, the carboxypeptidase II activity rose in the presence of 50 μg of chloramphenicol per ml to the same level as the control (Fig. 1). However, carboxypeptidase II activity dropped more rapidly in the presence of chloramphenicol. The absolute specific activity of carboxypeptidase II and the kinetics of the rise differed somewhat from those previously reported (4). The actual increase in specific activity and time of increase varied from experiment to experiment. These results indicate that protein synthesis is not responsible for the rise

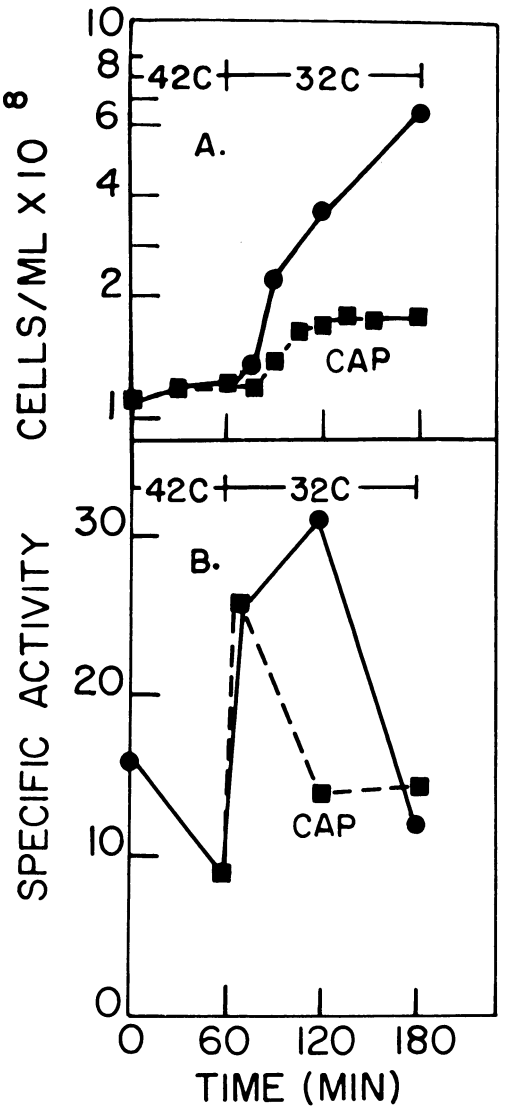

FIG. 1. *Cell division and carboxypeptidase II activity in the presence of chloramphenicol (CAP). (A) BUG 6 was grown in E medium plus glucose at 32°C until it had 0.2 unit of absorbancy at 585 nm and shifted to 42°C at zero time. At 60 min, the culture was shifted down to 32°C, and half received 50 μg of CAP per ml. Samples were taken for cell counting. (B) Cells were toluene treated and assayed for carboxypeptidase II activity.*

in carboxypeptidase II activity during cell division, unless the synthesis of either carboxypeptidase II itself or an activator of carboxypeptidase II is uniquely resistant to chloramphenicol inhibition of protein synthesis.

Regulation of carboxypeptidase II activity in nondividing and dividing cells of BUG 6. We investigated the differences between different preparations of nondividing and dividing cells of BUG 6 with respect to carboxypeptidase II activity. In addition, GDH and LDH (two cytoplasmic enzymes) and penicillinase, a periplasmic enzyme (12, 29), were assayed. The results of this investigation are shown in Table 1. Carboxypeptidase II activity was high in sonically disrupted samples from both nondividing and dividing BUG 6. This result, along with those of Fig. 1, shows that de novo synthesis of the carboxypeptidase II protein was not necessary for the increased activity of carboxypeptidase II during division.

Only 16% of the total carboxypeptidase II activity could be detected in toluenized, nondividing cells, whereas about 50% was measurable in toluenized, dividing cells. There were several possible explanations for this observation. (i) An inhibitor of carboxypeptidase II that was primarily present in nondividing cells became diluted out upon sonic oscillation and thus could no longer inhibit carboxypeptidase II in the completely disrupted extracts. (ii) Toluene treatment poorly disrupted the nondividing cells so that less UDP-MurNAc-tetrapeptide entered the cells. (iii) Toluene treatment liberated more enzyme from dividing cells than from nondividing cells.

There was a general increase in the permeability of the toluene-treated dividing cells as judged by the increased GDH and LDH activity relative to that of nondividing cells (Table 1). However, Table 2 shows that increasing the concentration of the substrate, UDP-MurNAc-tetrapeptide, above the usual 1.8 nmol per assay did not significantly increase enzyme activity in either nondividing or dividing toluene-treated cells. Thus, although the dividing cells were more permeable than nondividing cells, these changes did not seem sufficient to account for the changes in carboxypeptidase II activity.

Perhaps the most dramatic difference between carboxypeptidase II activity in nondividing cells and in dividing cells was in the amount of carboxypeptidase II enzyme released by Tris-EDTA treatment (Table 1). Little carboxypeptidase II activity was released from nondividing cells, wheras as much as one-third was released from dividing cells. In contrast, only minimal amounts (<3%) of the two cytoplasmic enzymes, GDH and LDH, were released from either nondividing or dividing cells. Penicillinase, another periplasmic enzyme, was released to a considerable extent from nondividing cells (43%) and to an even greater extent from dividing cells (71%).

Since Tris-EDTA treatment resulted in greater release of carboxypeptidase II activity

TABLE 1. *Enzyme activity of nondividing and dividing BUG 6 cells made permeable by different methods*[a]

Method for disruption of cells	Enzyme activity (U/100 ml of culture) of:							
	Carboxypeptidase II		Pencillinase		GDH		LDH	
	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells
Expt I								
Freeze-thaw treatment[b]	23	95	200	200	2,520	6,120		
Toluene treatment	31	132	ND[c]	ND	2,200	5,040		
Sonic oscillation	194	226	230	210	6,440	6,720		
Extract from Tris-EDTA treatment	6 (3.1)[d]	110 (48.7)	100 (43.5)	150 (71.4)	12 (0.2)	120 (1.8)		
Residue from Tris-EDTA treatment	154	77	240	140	6,560	6,560		
Expt II								
Freeze-thaw treatment	6	48					2	6
Toluene treatment	11	51					3	6
Sonic oscillation	75	120					45	63
Extract from Tris-EDTA treatment	1 (1.3)	30 (24.5)					1 (2.2)	2 (3.0)

[a] Dividing and nondividing cells were prepared as described in Materials and Methods.

[b] Freeze-thaw treatment was the same as toluene treatment minus toluene. Tris-EDTA residues were resuspended in 0.02 M Tris-hydrochloride (pH 8.0) and sonically oscillated. Activities are expressed as total units per 100 ml of culture.

[c] ND, Not determined.

[d] The numbers in parentheses refer to the percentage of total enzyme units released by the Tris-EDTA treatment.

from dividing cells, we decided to investigate whether the toluene treatment used to render cells permeable also resulted in the release of carboxypeptidase II from dividing cells. In fact, the use of toluene, under other conditions, has been reported to release certain periplasmic enzymes from *E. coli* (31). Carboxypeptidase II, LDH, and penicillinase were all released to different degrees by toluenization (Table 3). There was a 10-fold increase in the percentage of carboxypeptidase II released (3.4 to 35.1%) and only a 3-fold increase in the percentage of penicillinase released (20.5 to 59.4%) under dividing conditions as compared to nondividing conditions. Thus, the increase in carboxypeptidase II activity in dividing, toluene-treated cells can be largely accounted for by the actual release of enzyme from such cells by the toluene treatment itself, since enzyme outside the cell was shown to be more readily detected than the cell-associated enzyme (Table 1).

TABLE 2. *Effect of substrate concentration on carboxypeptidase II activity in toluene-treated cells of BUG 6*[a]

UDP-MurNAc-tetrapeptide (nmol/assay)	Enzyme activity (nmol hydrolyzed/15 min) in:	
	Nondividing cells	Dividing cells
1.8	0.05	0.27
3.6	0.05	0.30
7.2	0.08	0.33
10.8	0.08	0.28

[a] Nondividing and dividing cells of BUG 6 were prepared as described in Materials and Methods, except that dividing cells were harvested 20 min after the shift down to 32°C. Cells were toluene treated and assayed for carboxypeptidase II activity. There were 8.1 μg of protein in the assay of nondividing cells and 12.2 μg of protein in the assay for dividing cells.

The enzyme associated with the Tris-EDTA-treated residue from nondividing cells showed little activity (Table 4). Disruption of the Tris-EDTA-treated residues by ultrasonic oscillation resulted in a 14-fold increase in activity (Table 4). The Tris-EDTA residue from nondividing cells thus had much more "masked" enzyme activity than did the residue from dividing cells. Two possible explanations for this observation were: (i) a permeability barrier that prevented the uptake of substrate was present and was more effective in nondividing cells than in dividing cells or (ii) an inhibitor of carboxypeptidase II present in the residue became diluted out upon sonic oscillation, allowing detection of more total enzyme activity. The putative inhibitor would be present in higher concentrations in nondividing than in dividing cells. To test this hypothesis, we assayed carboxypeptidase II at different substrate concentrations in extracts prepared from dividing and nondividing cells. Table 5 shows that increasing the substrate concentration fivefold did not

TABLE 4. *Carboxypeptidase II activity in Tris-EDTA-treated samples of nondividing and dividing BUG 5 cells*[a]

Method of enzyme prepn	Enzyme activity (U/100 ml of culture) of:	
	Nondividing cells	Dividing cells
Extract from Tris-EDTA treatment	3	135
Residue from Tris-EDTA treatment	10	60
Sonically disrupted residue from Tris-EDTA treatment	138	87

[a] Nondividing and dividing cells of BUG 6 were grown and harvested as described in Materials and Methods. Activities are expressed as total units per 100 ml of culture.

TABLE 3. *Release of enzyme by toluene-treatment of nondividing and dividing BUG 6 cells*[a]

Source of enzyme	Enzyme activity (U/100 ml of culture) of:					
	Carboxypeptidase II		Penicillinase		LDH	
	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells
Toluene extract	9	82	32	193	5	42
Toluene residue	152	87	110	72	247	272
% Released[b]	3.4	35.1	20.5	59.4	0	0

[a] Nondividing and dividing cells of BUG 6 were prepared as described in Table 1. Cells were toluene treated and centrifuged for 5 min at 8,700 × *g* in a Beckman 152 microfuge. The supernatant solution was decanted, and the pellet was resuspended in the same volume of 0.02 M Tris-hydrochloride (pH 8.0) and sonically disrupted. Activities are expressed as total units per 100 ml of culture.

[b] The percentage of enzyme released has been corrected for lysis based on the percentage of LDH released.

TABLE 5. *Effect of substrate concentration on carboxypeptidase II activity in undisrupted residues of Tris-EDTA treated cells*[a]

Source of residue	Substrate concn (nmol/20 μl)	^{14}C-D-alanine released (cpm)
Expt 1		
Nondividing cells	2	882
	10	940
Dividing cells	2	2,254
	10	2,741
Expt 2		
Nondividing cells	2	176
	10	258
Dividing cells	2	2,628
	10	4,266

[a] BUG 6 was grown in E medium plus glucose at 32°C until it had a measurement of 0.3 units of absorbancy at 585 nm. Cells were shifted to 42°C for 75 min (nondividing) and then to 32°C for 15 min (dividing). Cells were harvested and treated with Tris-EDTA, and the residues were assayed directly for carboxypeptidase II activity.

significantly increase carboxypeptidase II activity in nondisrupted Tris-EDTA residues from either dividing or nondividing cells, suggesting that the substrate was not limiting.

Since permeability changes are insufficient to explain the very low activity detectable in the nondividing cells after Tris-EDTA treatment, it is concluded that an inhibitor of the enzyme may be present in nondividing cells. Further evidence for this hypothesis would involve isolation and characterization of the putative inhibitor.

Release of carboxypeptidase II from other cell division mutants by Tris-EDTA. To determine if the relationship between cell division and the release of carboxypeptidase II by Tris-EDTA was a phenomenon peculiar to BUG 6 or a general property of the division process, two other division mutants of *E. coli* K-12, $P_{4\times8}ts_{84}$ (28) and AX655 (2), were studied. Both mutants grew well and formed filamentous, nondividing cells at the restrictive temperature (42°C). Thirty minutes after a shift down to the permissive temperature (28°C), the cells divided without lysis or cessation of growth. The release of carboxypeptidase II and penicillinase by Tris-EDTA treatment of nondividing and dividing cells of $P_{4\times8}ts_{84}$ and AX655 was therefore determined.

The release pattern of carboxypeptidase II and penicillinase from nondividing and dividing cells of $P_{4\times8}ts_{84}$ was similar to the release pattern in BUG 6 (Table 6). AX655 showed less of an increase in the amount of carboxypeptidase II released at the time of division (8.5 to 18.2%). However, microscopic examination of AX655 revealed that filaments were present in cultures grown at 28°C. The division process in this organism may be somewhat impaired, even at the permissive temperature. The percentage of carboxypeptidase II released by Tris-EDTA treatment in the wild-type cells, AB1157 and D11, was relatively unaffected by temperature shifts (Table 7). Thus, the enhanced release of carboxypeptidase II by Tris-EDTA in temperature-sensitive division mutants at the

TABLE 6. *Release of carboxypeptidase II and penicillinase from division mutants by Tris-EDTA treatment*[a]

Source of enzyme	Enzyme activity (U/100 ml of culture) of:			
	Carboxypeptidase II		Penicillinase	
	Nondividing cells	Dividing cells	Nondividing cells	Dividing cells
$P_{4x8}ts_{84}$ extract	11 (9.2)	72 (40.7)	90 (64.3)	155 (72.1)
$P_{4x8}ts_{84}$ residue	108	105	50	60
AX 655 extract	4 (8.5)	14 (18.2)	30 (30)	70 (48.5)
AX655 residue	47	63	70	85
BUG 6 extract	4 (2.3)	78 (40.9)	40 (30.8)	90 (56.3)
BUG 6 residue	149	113	90	70

[a] Cells were grown in E medium plus glucose at 28°C until they had a measurement of 0.2 to 0.3 units of absorbancy at 585 nm, shifted to 42°C for 60 min, and shifted down to 28°C for 30 min. Cells were treated with Tris-EDTA, and the residue was resuspended in 0.02 M Tris-hydrochloride (pH 8.0) and sonically disrupted. Activities are expressed as total units per 100 ml of culture. The numbers in parentheses refer to the percentage of total enzyme units released by the Tris-EDTA treatment.

TABLE 7. *Lack of effect of temperature shift on carboxypeptidase II release by Tris-EDTA in wild-type cells*[a]

Source of enzyme	Enzyme activity (U/100 ml of culture) at:		
	42°C, 60 min	28°C, 15 min	28°C, 30 min
AB1157 extract	33 (37.5)	31 (35.2)	56 (40.0)
AB1157 residue	55	57	84
D11 extract	17 (22.4)	22 (22.0)	26 (24.8)
D11 residue	59	78	79

[a] Cells were grown in E medium plus glucose at 28°C until they had a measurement of 0.2 to 0.4 units of absorbancy at 585 nm, shifted to 42°C for 60 min, and shifted to 28°C. Cells were harvested after 60 min at 42°C, harvested 15 and 30 min after the shift down to 28°C, and treated with Tris-EDTA. Residues were resuspended in 0.02 M Tris-hydrochloride (pH 8.0) and sonically disrupted. Activities are expressed as units per 100 ml of culture. The numbers in parentheses refer to the percentage of total enzyme units released by the Tris-EDTA treatment.

time of division is a property of the division process and not of the temperature shift.

Purification of carboxypeptidase II from BUG 6. Purification of carboxypeptidase II was complicated by the instability of the enzyme, especially at low protein concentrations. The loss of enzyme activity in dilute samples could be partially prevented by 4 mM dithiothreitol or 0.2 mg of bovine serum albumin per ml. In the concentrated samples, these additions had either no effect or were inhibitory. Unless otherwise stated, 4 mM dithiothreitol was included in all buffers and reagents.

Carboxypeptidase II could be separated from the bulk of the proteins in a Tris-EDTA extract from dividing cells of BUG 6 by DEAE-cellulose column chromatography. Carboxypeptidase II adsorbed strongly to the DEAE-cellulose column, being eluted only at salt concentrations significantly higher than necessary to elute bulk protein (Fig. 2). DEAE-cellulose column chromatography usually gave a 5- to 10-fold purification of carboxypeptidase II.

The molecular weight of the native carboxypeptidase II enzyme was estimated by Sephadex G-200 column chromatography (Fig. 3). Carboxypeptidase II always eluted in one peak. By comparison to hemoglobin and cytochrome *c*, the native carboxypeptidase II molecule was estimated to have a molecular weight of about 86×10^3.

A purification scheme for carboxypeptidase II that utilized DEAE column chromatography followed by Sephadex G-200 column chromatography was devised. The results of this purification are shown in Table 8. The second DEAE

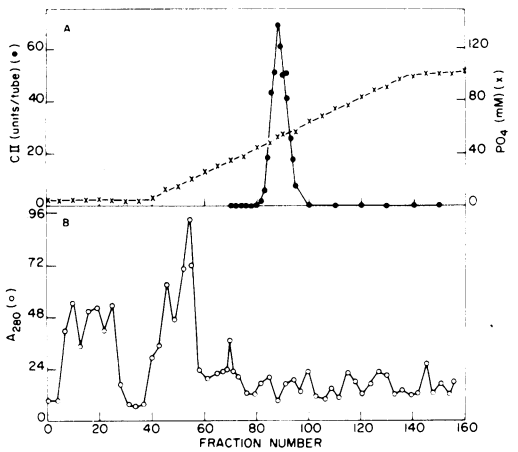

FIG. 2. *DEAE-column chromatography of carboxypeptidase II. Tris-EDTA extract was prepared from 20 liters of dividing BUG 6 cells, as described in Materials and Methods. This extract was dialyzed overnight in tubing prepared as described in Materials and Methods until the conductivity was less than that of 2 mM potassium phosphate (pH 7.2). The extract was then applied to a column (3 by 9 cm) of DE52. A 1-liter gradient of 2 to 100 mM potassium phosphate (pH 7.2) was applied with a Buchler pump. Nine-milliliter fractions were collected. Carboxypeptidase II (CII) activity is expressed as total units per fraction. A_{280}, units of absorbancy at 280 nm.*

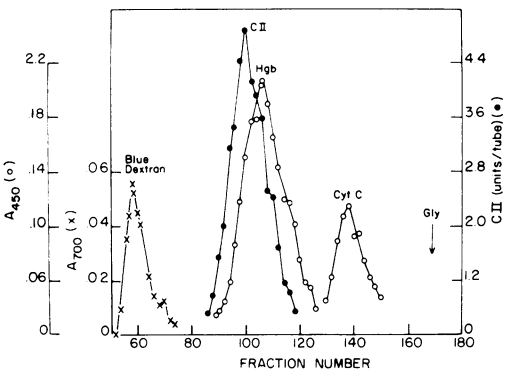

FIG. 3. *Estimation of the molecular weight of carboxypeptidase II by chromatography on Sephadex G-200. Tris-EDTA extract was prepared from dividing BUG 6, grown as described in Materials and Methods. A 4-ml portion of this extract, 2 mg each of Blue Dextran (Pharmacia), hemoglobin tetramer (Hgb; molecular weight, 64,500) and cytochrome c (CytC; molecular weight 11,700), and 50 mg of glycine (Gly) were chromatographed on a Sephadex G-200 (Pharmacia) column as described in Materials and Methods. Blue Dextran was located by measuring its absorbancy at 700 nm; Hgb and CytC were located by measuring their absorbancy at 430 nm. Glycine was located by ninhydrin spray on 20 μl of each sample spotted on Whatman 3MM paper. Carboxypeptidase II activities are expressed as total units per fraction.*

TABLE 8. *Purification of carboxypeptidase II*[a]

Step	Total units	Protein (mg)	Sp act	Purification (-fold)
1. Whole cell extract	94,700	5,300	17.8	
2. Tris-EDTA extract	26,600	225	118.1	6.6
3. DEAE column I	22,200	21.6	1,027.8	57.7
4. DEAE column II	3,500 (theoretical yield, 11,100)	4.7	739	41.5
5. Sephadex G-200	222 (theoretical yield, 673)	0.6	239	13.4

[a] Carboxypeptidase II was purified as described in Materials and Methods.

column was necessary to concentrate the enzyme for application to the Sephadex column. Despite the presence of 4 mM dithiothreitol, there was a loss of two-thirds of the enzyme activity in both steps 4 and 5. Thus, the specific activity measurements were not meaningful after step 4.

To ascertain the purity of the carboxypeptidase II enzyme after DEAE-cellulose and Sephadex G-200 chromatography, the sample was analyzed by SDS-polyacrylamide electrophoresis. Figure 4 shows that most of the protein from the final purification step was located in a single band. This band co-migrated with ovalbumin (molecular weight, 43×10^3). This suggests that carboxypeptidase II, in its native form, was a dimer.

To biochemically confirm that carboxypeptidase II was released by Tris-EDTA treatment of dividing cells, but not by Tris-EDTA treatment of nondividing cells, extracts from nondividing and dividing cells were compared with purified carboxypeptidase II protein by SDS-polyacrylamide gel electrophoresis. It should be noted that the amount of protein released by Tris-EDTA treatment of nondividing cells was only 10% of the amount of protein released from dividing cells. The SDS-polyacrylamide gel analysis revealed that the carboxypeptidase II protein was present in Tris-EDTA extracts

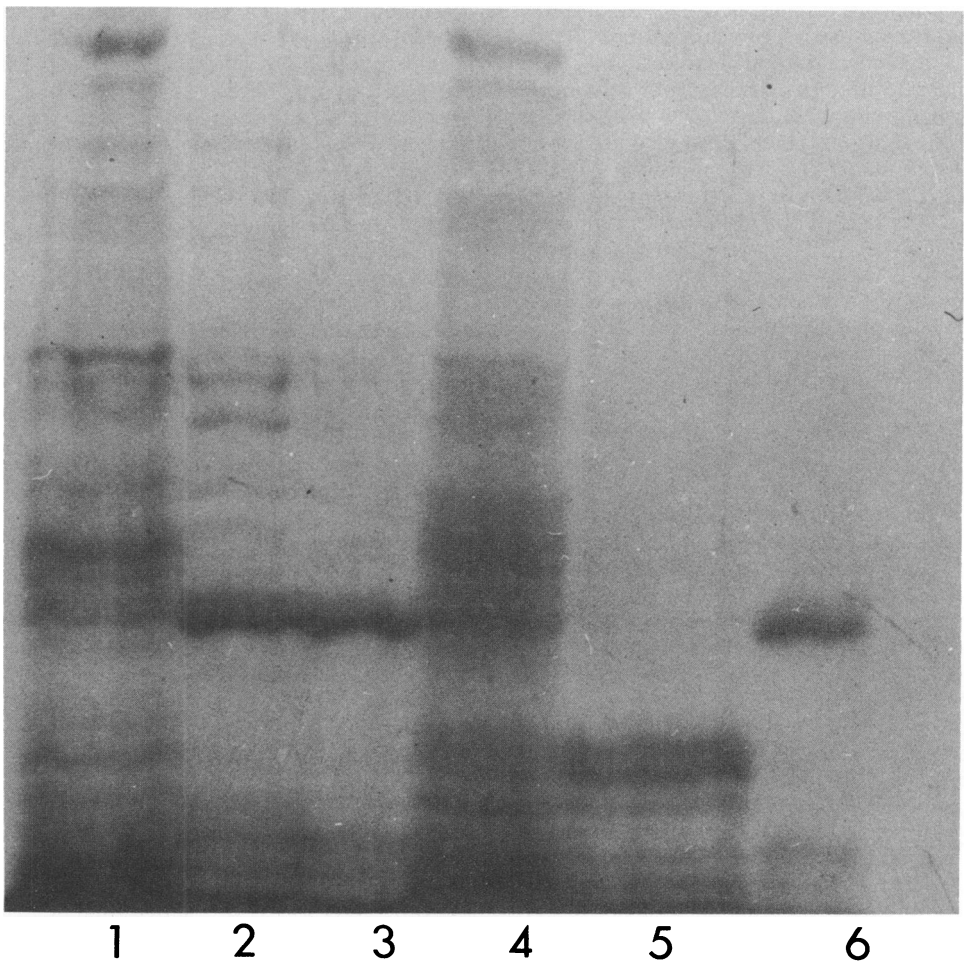

FIG. 4. *Proteins released by Tris-EDTA treatment of nondividing and dividing cells. SDS-polyacrylamide gel electrophoresis was for 120 min at 80 V. The source of the samples and the amount applied were (from left to right): (1) Tris-EDTA extract from purification step 2 (Table 8), 30 μg; (2) DEAE-cellulose column extract from step 3 (Table 8), 12 μg; (3) Sephadex G-200 column extract from step 5 (Table 8), 9.6 μg; (4) Tris-EDTA extract from dividing BUG 6 grown as described in Materials and Methods, 30.5 μg; (5) Tris-EDTA extract from nondividing BUG 6 grown as described in Materials and Methods, 52.5 μg; (6) same as number (3). The direction of migration was from top to bottom.*

from dividing cells and not detectable in Tris-EDTA extracts from nondividing cells (Fig. 4). Furthermore, much less than 10% of the high-molecular-weight proteins and about 10% of most of the low-molecular-weight proteins were extracted from nondividing cells. This suggests that a barrier which prevents the Tris-EDTA-induced release of periplasmic proteins exists in nondividing cells. Thus, a surface structure that is recognizable in Tris-EDTA-treated cells as a sieve that retains periplasmic proteins appears to exist during the period between cell divisions.

DISCUSSION

We have investigated the regulation of carboxypeptidase II during the cell cycle using the temperature-sensitive division mutant BUG 6 (24) as a model system. The activity of this enzyme in different extracts from cells grown under nondividing conditions (1 h at 42°C) or dividing conditions (15 min after a shift down to 32°C) was measured. We found that the division-associated increase in carboxypeptidase II activity occurred in the absence of protein synthesis (Fig. 1). The finding that all sonically disrupted cells had high carboxypeptidase II activity (Table 4) provided further evidence that de novo protein synthesis was not required for the division-associated rise in activity. This finding is similar to those presented in a recent study by Mirelman et al. (21) on the activity of the terminal D-alanine-removing enzyme, carboxypeptidase I, in the temperature-sensitive filament former, PAT 84. Cells rendered permeable by ether had higher carboxypeptidase I activity under dividing conditions (30°C) rather than under nondividing conditions (42°C). However, cells which had been both ether treated and sonically disrupted had the highest carboxypeptidase I activity. Thus, the division-associated change in carboxypeptidase I in PAT 84 was not a result of changes in total amount of enzyme protein. The increase in carboxypeptidase I at the time of division in PAT 84 contrasts with our previous finding of unchanged carboxypeptidase I activity during the cell cycle in cells of D11-1 sized by sucrose gradient centrifugation (4). In experiments with BUG 6, we have sometimes observed a small increase in carboxypeptidase I activity at division (data not shown). This increase was always considerably less than the increase in carboxypeptidase II activity. The incomplete sizing of cells by the sucrose gradient centrifugation method would have obscured a small rise in carboxypeptidase I activity.

The high level of carboxypeptidase II in sonically disrupted, nondividing cells contrasted to the minimal level observed in toluene-treated whole cells (Table 1). This suggests that either an inhibitor of carboxypeptidase II was present in nondividing cells or that the substrate might be limiting. Although there was a general increase in permeability occurring at division (Table 1), the enzyme was close to saturation with substrate (Table 2). However, a complication of the studies with toluene-treated cells is that toluene treatment liberates carboxypeptidase II from the cell and more enzyme is liberated from dividing than from nondividing cells (Table 3). Thus, to further investigate the question of permeability differences and/or the presence of an inhibitor in nondividing cells, we assayed the enzyme activity remaining in undisrupted Tris-EDTA residues, using different concentrations of substrate. The results suggested that cell-associated enzyme was essentially saturated with substrate. Thus, the presence of an inhibitor seems to be the most likely explanation for the low carboxypeptidase II activity in nondividing cells. We have attempted to demonstrate more directly the presence of an inhibitor in the Tris-EDTA residues by sonically disrupting such a preparation in as concentrated a volume as possible. However, even this preparation must represent a large dilution of the periplasmic volume, and hence it is not surprising that the "concentrate" was active and that further dilution resulted in only 33% additional activity (data not shown). There was no strong evidence for an inhibitor or an activator under these conditions. The regulation of carboxypeptidase II activity during the cell cycle appears complex and will perhaps be best resolved through studies using purified enzyme and searching for periplasmic or membrane components that fluctuate in concentration and which may modulate enzyme activity.

A striking finding of these studies is that carboxypeptidase II was more readily released by Tris-EDTA treatment and by toluene treatment of dividing cells of BUG 6 than by Tris-EDTA or toluene treatment of nondividing cells of BUG 6 (Tables 1 and 3). The increase in releasability of carboxypeptidase II by Tris-EDTA treatment at the time of division was confirmed by SDS-polyacrylamide gel analysis of Tris-EDTA extracts from nondividing and dividing cells (Fig. 4), which demonstrated that carboxypeptidase II was absent in Tris-EDTA extracts from nondividing cells and present in Tris-EDTA extracts from dividing cells (Fig. 4). In fact, most of the periplasmic proteins were reduced by 90% in the extracts from nondividing cells. These results indicate that the cell envelope of *E. coli* under nondividing conditions differs from that of cells in the act of

division, since it acts as a molecular sieve to prevent the Tris-EDTA-induced release of periplasmic protein molecules. This molecular sieving is observed only when part of the outer membrane is removed by Tris-EDTA treatment and is not observed in untreated cells in which periplasmic proteins are retained by the intact outer membrane. The division-associated releasability of carboxypeptidase II is superficially similar to Shen and Boos' finding that the periplasmic galactose-binding protein is present only in osmotic shock fluid from dividing cells (27). However, the presence or absence of the galactose-binding protein in the osmotic shock fluid was correlated with its synthesis.

The nature of this barrier in nondividing cells is unknown. Morphological studies of cells of *E. coli* collected throughout the division cycle reveal a bleb that consists of an evagination of the outer membrane in the septal region just prior to division (6, 7). Furthermore, a class of mutants of *Salmonella typhimurium* that leak certain periplasmic enzymes also exhibit these blebs, which are localized in either the septal or the polar region (34, 35). Studies on minicells of *E. coli*, which are the product of an abnormally situated septum (1) and should therefore contain a high proportion of septal material, indicate that certain periplasmic enzymes may be localized in the septal region (9). Thus, it is possible that the barrier to the release of periplasmic proteins is absent in the region of the septal blebs.

The outer membrane is anchored to the murein layer by the Braun lipoprotein, which is covalently attached to the murein at the carboxyl terminus of the protein (5). About two-thirds of the lipoprotein molecules are free in the outer membrane (14). At division, there is an increase in the amount of free lipoprotein incorporated into the outer membrane in *E. coli* (16). The periplasmic leaky mutants of *S. typhimurium* have a decrease in the bound form of lipoprotein as compared to the parent strain (35). It therefore seems possible that the covalent linkage of murein to lipoprotein forms the "barrier" that we detect in nondividing cells this "barrier" is absent in the septal region. It is also possible that the barrier is the murein sacculus itself and that the barrier breaks down more readily in dividing cells because of the increased autolytic activity. This fluctuation in outer membrane properties may well be without physiological significance and merely be incidental to the alterations in free and bound lipoprotein (16), which in turn might be brought about by the division-associated rise in carboxypeptidase II activity. Alternatively, the alteration in membrane properties may cause the release of the hypothetical inhibitor, thus triggering the rise in carboxypeptidase II activity.

Carboxypeptidase II may prevent the linkage of diaminopimelic acid in the murein layer to the lipoprotein by removing the subterminal D-alanine residue before it can be utilized to drive the hypothetical murein-lipoprotein cross-linking reaction (4, 14), or its role may be more subtle. The increase in free lipoprotein at the time of division (16, 35) and our evidence that carboxypeptidase II is more active in dividing cells suggest that carboxypeptidase II may prevent the association of the outer membrane and the murein layer at the time of septum formation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant no. AI 05090 from the National Institute of Allergy and Infectious Diseases. Barbara D. Beck was a predoctoral trainee supported by Public Health Service grant no. GM 01136 from the National Institute of General Medical Sciences. The skillful assistance of Cynthia Neumann is gratefully acknowledged.

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