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Effects of Proline Analogues on the Formation of Alkaline Phosphatase in *Escherichia coli*

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Two of the four proline analogues tested for their effect on the formation and activity of *Escherichia coli* alkaline phosphatase were able to substitute for proline in protein synthesis in a proline auxotroph. One of these, 3,4-dehydroproline, effectively replaced proline and led to formation of an active enzyme under conditions where no proline was present in the polypeptides. Substitution of azetidine-2-carboxylate for proline prevented active enzyme formation, producing instead altered monomeric forms of the alkaline phosphatase. These were detected with antibodies specific to denatured forms of the enzyme, and they were also characterized, together with cellular proteins, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Alkaline phosphatase, as well as several other proteins, is localized exterior to the bacterial cell cytoplasm in the periplasmic space. In the presence of azetidine-2-carboxylate, a substantial number of these periplasmic proteins retain their specific site of localization, and the denatured subunits of alkaline phosphatase were only detected in the periplasmic fraction of the cell. Thus, secretion of these proteins does not appear to require a high degree of specificity in the native structure of the polypeptide chain. The analogues 4-allohydroxyproline and 4-thiazolidine carboxylate were unable to substitute for proline in protein synthesis but they inhibited growth of *E. coli*.

An important stage in the formation of the alkaline phosphatase of *Escherichia coli* (EC 3.1.3.1.) is the transport of the protein from its site of synthesis in the cytoplasm to its site of function in the periplasm, a region of the bacterial cell exterior to the cytoplasmic membrane. From studies with several alkaline phosphatase-negative mutants that synthesized antigenically related forms of the enzyme, we had determined that the protein was probably transported in the form of the subunit *prior* to dimerization and activation by metal (15). These latter two additional reactions are essential for production of an active enzyme. Support for this model of transport of the subunit came from observations that *E. coli* spheroplasts that were able to make protein but unable to form active alkaline phosphatase secreted subunits of this protein into the spheroplast medium (14). Mechanisms for the transport of large polypeptides across bacterial membranes have not been described, but any system of transport must possess a high degree of specificity because only a small fraction of bacterial proteins are localized outside the cytoplasm. We have attempted to determine whether the specificity for alkaline phosphatase transport lies in the secondary or tertiary structure of the polypeptide chain. Because protein structure depends upon the specific amino acid sequence in the polypeptide, we altered the primary structure with analogues of amino acids that were able to substitute for the naturally occurring amino acid in protein synthesis. Previous experiments with two histidine analogues (1,2,4-triazole-3-alanine and 2-methyl histidine) and an arginine analogue (canavanine) showed that alkaline phosphatase polypeptides that had been altered to the extent that they could not dimerize and form active enzyme could, nevertheless, be transported almost quantitatively into the periplasmic space (1, 18, 19).

In a further attempt to block the transport process, we have extended these studies to four analogues of proline: 4-thiazolidine carboxylate, 4-allo-hydroxyproline (*cis*-hydroxyproline), azetidine-2-carboxylate, and 3,4-dehydro-

proline. With the exception of *cis*-hydroxyproline, all of these have been reported to inhibit growth of *E. coli*, and the inhibition was reversed by adding proline to the growth medium (5, 6, 22). These experiments were performed with strains prototrophic for proline, and it was not possible to determine whether inhibition was caused by a block in proline biosynthesis or by incorporation of the analogue in polypeptides. Such inhibition only provides unambiguous data that the analogue has entered the cell.

Our studies were aimed at testing those analogues capable of replacing proline in protein, and we therefore isolated a proline auxotroph. Only the analogues azetidine-2-carboxylate and 3,4-dehydroproline could support protein synthesis when proline was absent; thus, these compounds could substitute for proline in polypeptides. Formation of alkaline phosphatase protein continued in the presence of the analogues, but active enzyme was formed only in the presence of 3,4-dehydroproline. Substitution of azetidine-2-carboxylate for proline inhibited the assembly and metal activation processes and led to an accumulation of altered peptides in the periplasmic space. These results are similar to the previous studies and further strengthen the conclusion that disruption of the secondary and tertiary structure of the alkaline phosphatase polypeptide does not affect its transport.

MATERIALS AND METHODS

Bacterial strains. Proline auxotrophs of *E. coli* W3747 (ATCC 27256) and CW3747 (ATCC 27257) were isolated by procedures described previously (18). In strain W3747 Pro$^-$, alkaline phosphatase is repressed during exponential growth phase and derepression occurs when inorganic phosphate is removed from the medium. CW3747 Pro$^-$ is a strain, derived from W3747 Pro$^-$, that synthesizes alkaline phosphatase constitutively (i.e., during exponential growth phase). The growth medium was the same as described in earlier experiments (13).

Preparation of cytoplasmic and periplasmic fractions. Cells were grown and spheroplasts were prepared with lysozyme as described previously (17), with the following modifications. Cells from a 1-liter culture were harvested, washed with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 8.0, and resuspended in 20 ml containing 12% sucrose and 0.3 M Tris-chloride, *p*H 8.0. One milliliter of lysozyme (2 mg/ml) was added, followed 2 min later by 0.2 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA). Spheroplast formation was measured by noting the change in absorbance (540 nm) when a 10-μliter sample was added to 1.0 ml of water. The conversion of more than 90% of the cells to spheroplasts generally occurred within 15 to 20 min, and 0.2 ml of M magnesium acetate was added at that time. The protein released from the spheroplasts (termed "periplasmic fractions") was separated from the spheroplasts by high-speed centrifugation and was dialyzed against 10 mM Tris-chloride, *p*H 8.0, 1 mM magnesium acetate at 4 C for 16 hr. The dialysis bag was transferred to a solution of 20% Carbowax 6000 (Union Carbide) in the same buffer to concentrate the fraction about 10-fold. The spheroplasts were resuspended in a buffer containing 10 mM Tris-chloride (*p*H 7.4), 1 mM magnesium acetate, and 10 μM zinc chloride, sonically disrupted, and centrifuged for 90 min at 50,000 $\times$ *g*. The supernatant fraction was analyzed and is termed "cytoplasmic fraction."

Precipitation with antibodies. Antibodies to a fragment (22,000 daltons) of alkaline phosphatase isolated after treating the pure enzyme with cyanogen bromide were prepared by the same method used for the preparation of antibodies directed against intact alkaline phosphatase subunits (43,000 daltons) (12). The preparation is referred to as the antiphosphatase fragment antibody. It is able to form precipitates with alkaline phosphatase subunits but not with intact dimeric enzyme (M. J. Schlesinger, *unpublished data*). When these antibodies were incubated with the periplasmic fraction prepared from an amber mutant (U·18) that could not make that part of the polypeptide used as the antigen, the amount of ^{14}C-protein precipitated was no greater than that measured when a completely unrelated set of antibodies was used to form a precipitating reaction. "TN antibodies" are from rabbits injected with trinitrophenyl-γ-globulin and have been described previously (14). The precipitin reactions were carried out as described previously (19).

Analysis of proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Gels were prepared as described by Viñuela, Algranati, and Ochoa (23) except 7.5% (w/v) acrylamide and 0.1 M sodium phosphate buffer, *p*H 7.0, were used and mercaptoethanol was omitted. The samples were prepared in 15% sucrose, 0.1% SDS and 0.01 M sodium phosphate, *p*H 7.0. Electrophoresis was carried out at room temperature in an analytical apparatus manufactured by Hoeffer Scientific Instruments, at a constant voltage and initial current of 8 ma/gel for 4.5 hr. After electrophoresis, the gels were removed from the tubes with gentle air pressure and placed in a groove in a stainless-steel block, previously cooled in a dry ice-acetone bath. The block has a series of transverse slits approximately 1.8 mm apart throughout its length. Frozen gels were sliced into approximately 40 slices with a razor blade. Each gel slice was placed in a scintillation vial, and 0.5 ml of concentrated ammonium hydroxide was added. After 16 hr at room temperature, 0.1 ml of water was added followed by 10 ml of scintillant containing 9.3 g of 2,5-diphenyloxazole and 30 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter and 625 ml of toluene and 375 ml of methyl Cellosolve.

Chemicals. L-Azetidine-2-carboxylate was from Aldrich, ^{14}C-L-azetidine-2-carboxylate from Calbiochem, 4-thiazolidine carboxylate from Eastman, allo-4-hydroxyproline from Calbiochem, and 3,4-

dehydroproline was kindly supplied by Annette Baich. ^{14}C-leucine was from New England Nuclear Corp., and ^{3}H-leucine and ^{3}H-proline from Schwartz-Mann.

Enzyme assay. The assay for alkaline phosphatase with *p*-nitrophenyl phosphate as substrate has been described (13).

Measurement of radioactivity. The measurement of ^{14}C-protein was carried out as described by Attias, Schlesinger, and Schlesinger (1).

RESULTS

Effect of proline analogues on cell growth, protein synthesis, and alkaline phosphatase. Replacement of proline by the analogues *cis*-hydroxyproline or thiazolidine carboxylate resulted in immediate cessation of growth, protein synthesis, and alkaline phosphatase production in a proline auxotroph of *E. coli* (Fig. 1, series A). With azetidine carboxylate, protein synthesis was 10% that measured with proline, but active alkaline phosphatase formation stopped immediately (Fig. 1, series B). The limited amount of 3,4-dehydroproline available restricted the concentration used in experiments with this analogue to one-tenth that of proline. Even under these conditions this analogue could support significant protein synthesis and alkaline phosphatase formation (Fig. 1, series B).

Incorporation of 3,4-dehydroproline into protein. Dehydroproline was shown to support protein synthesis, but there was the possibility that the analogue was converted to proline before incorporation into protein. If the analogue was directly incorporated, the protein formed in the presence of the analogue should possess properties distinct from those of the normal enzyme. Alkaline phosphatase was isolated in partially purified form from W3747 Pro$^-$ cells starved for P_i with 3,4-dehydroproline as the source of proline. This enzyme preparation lost 85% of its original activity after 30 min at 90 C, whereas purified proline-containing alkaline phosphatase (16) lost only 45% of its activity under identical conditions. Similar data has been reported by Fowden et al. (4) for an alkaline phosphatase in which 80% of the proline residues were estimated to be replaced by 3,4-dehydroproline.

Localization and nature of protein synthesized in the presence of azetidine carboxylate. When ^{14}C-leucine was added to an exponentially growing culture of *E. coli* W3747 Pro$^-$ in the presence of proline, 8% of the total soluble ^{14}C-protein was released by spheroplast formation, i.e., this amount represents periplasmic proteins (Table 1). This same distribution of periplasmic proteins was obtained in a culture of the auxotroph when the isotope was added after the cells had been washed free of proline and suspended in medium containing azetidine carboxylate. Thus, the quantitative distribution of protein between cytoplasm and periplasm was not significantly altered by addition of this analogue.

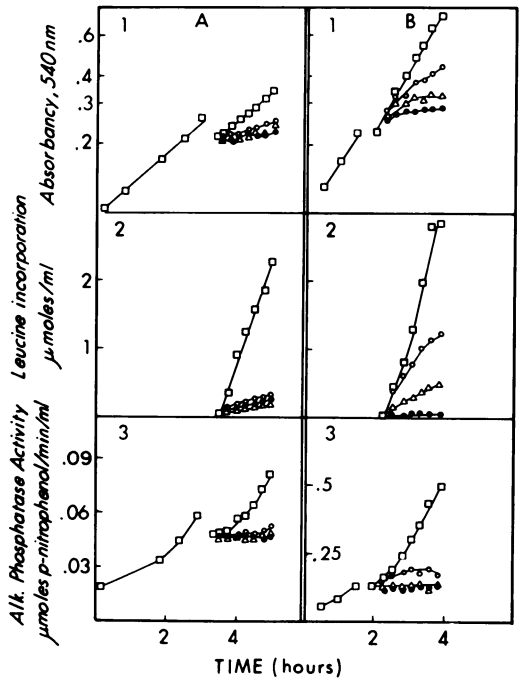

FIG. 1. *Effect of proline analogues on cell growth, protein synthesis, and alkaline phosphatase production in E. coli. Exponentially growing cells (CW3747 Pro$^-$) were harvested by centrifugation and resuspended in an equal volume of proline-free medium. The culture was divided into four 100-ml fractions which were incubated at 37 C for 5 min before addition of 0.2 mM proline (□), the proline analogues (see below), or no amino acid (●). A 10-μCi amount of radioactive leucine (0.05 mCi/0.0251 mg) was added to the flasks 2 min later to a final concentration of 0.115 mM. Samples were removed at the indicated times for analyses: (1) cell growth, (2) protein synthesis, (3) alkaline phosphatase activity. Series A: ○; 0.2 mM cis-hydroxyproline; △, 0.2 mM 4-thiazolidine carboxylate. Series B: ○, 0.02 mM 3,4-dehydroproline; △, 0.2 mM azetidine-2-carboxylate.*

Derepression of alkaline phosphatase formation in this strain, either by starvation for P_i or by a mutation in a regulator gene, increased the amount of total periplasmic protein some threefold to a value of 25% of the total soluble protein. Almost one-third of this increase could be attributed to alkaline phosphatase alone (Table 1 and reference 14). Substitution

of azetidine carboxylate for proline both inhibited this increase in periplasmic protein (Table 1) and altered the pattern of cytoplasmic and periplasmic proteins revealed in an SDS gel electropherogram (Fig. 2, panels 1 and 2). The reduced amount of periplasmic protein could not be attributed to secretion of altered polypeptides into the cell media. No significant amount of ^{14}C-protein was secreted during growth in azetidine carboxylate, nor was there a difference detected in the SDS-polyacrylamide gel profile of that small amount of protein in the media (Fig. 2, panel 3).

These results with azetidine carboxylate should be compared with data obtained with 3,4-dehydroproline employed at one-tenth the concentration used for proline. With the latter analogue, alkaline phosphatase was formed as an active enzyme (*see above*), and the pattern of periplasmic proteins observed in SDS-polyacrylamide gels was qualitatively similar to that found in cells grown with proline and distinct from that in cells grown with azetidine carboxylate (Fig. 2, panel 4). However, panel 4 in Fig. 2 shows a significant reduction in the amount of alkaline phosphatase (peak a) as well as three or four other proteins formed in the presence of 3,4-dehydroproline. In addition, cells constitutive for alkaline phosphatase that were grown in a medium containing limiting levels of 3,4-dehydroproline formed only 10% of their soluble protein as periplasmic material compared to values of 25% for proline-grown cells and 7% for azetidine carboxylate-grown cells.

The mechanisms for the change in relative distribution of protein between cytoplasm and periplasm when the two analogues are added to derepressed cells are not understood. The two analogues differ greatly in their qualitative effects, and azetidine carboxylate did not alter the quantitative distribution of protein between periplasm and cytoplasm in cells repressed for alkaline phosphatase formation (Table 1). Thus, it is unlikely that the reduction in periplasmic protein effected by the analogues is related simply to alterations in a transport process. Possibly, the decreased protein synthesis caused by azetidine carboxylate and the limiting level of 3,4-dehydroproline have affected the induction of alkaline phosphatase and other proteins that may be induced under the same conditions. We have observed that at least three other periplasmic proteins are quantitatively altered under conditions of phosphatase derepression.

Formation of alkaline phosphatase subunits containing azetidine-2-carboxylate. Despite the inhibition of periplasmic protein formation by azetidine carboxylate, a signifi-

TABLE 1. *Distribution of protein between cytoplasm and periplasm of Escherichia coli*[a]

Strain	Growth conditions	Proline source	^{14}C- or ^{3}H-protein counts/min		Protein in periplasm (% total soluble protein)	Alkaline phosphatase (% total soluble protein in the cell)[b]
			Cytoplasm	Periplasm		
W3747	P_i-starved	Azetidine carboxylate	1.9×10^5	3.1×10^4	14	(0.2)
W3747	P_i-starved	Proline	1.3×10^6	3.2×10^5	20	6.3
W3747	Exp	Azetidine carboxylate	8×10^6	7.1×10^5	8	(<.05)
W3747	Exp	Proline	1.4×10^6	1.2×10^5	8	<.01
CW3747	Exp	Azetidine carboxylate	1.7×10^5	1.3×10^4	7	(.1-.2)
CW3747	Exp	Proline	1.25×10^5	4.5×10^4	26	5.5

[a] All strains in these experiments were auxotrophic for proline. Incorporation of the radioactive amino acid was carried out for 90 min under the conditions described in the legend to Fig. 1. Cytoplasmic and periplasmic fractions were prepared as described in Materials and Methods. The following isotopes were used: 50 μCi of ^{14}C-azetidine carboxylate (5-15 mCi/mmole) per liter of W3747, P_i-starved; 20 μCi of ^{3}H-proline (1-3 Ci/mmole) per liter of exponentially growing (Exp) and P_i-starved; 20 μCi of ^{14}C-leucine (250 mCi/mmole) per liter of W3747, Exp in presence of azetidine carboxylate and 10 μCi of this isotope to 100 ml of CW3747, Exp in presence of azetidine carboxylate; 10 μCi of ^{3}H-leucine (2 Ci/mmole) to 100 ml of CW3747, Exp in presence of proline. Alkaline phosphatase was determined from the counts per minute in the alkaline phosphatase peak after SDS-polyacrylamide gel electrophoresis (cf. Fig. 2) or from the amount precipitated by the antiphosphatase fragment antibodies (in parentheses). Greater than 90% of the cells were converted to spheroplasts as measured by the release of alkaline phosphatase, and there was less than 5% lysis of the spheroplasts as measured by the release of glucose-6-phosphate dehydrogenase (13).

[b] Data were obtained from analyses of the periplasmic fractions but have been corrected for total soluble cell protein.

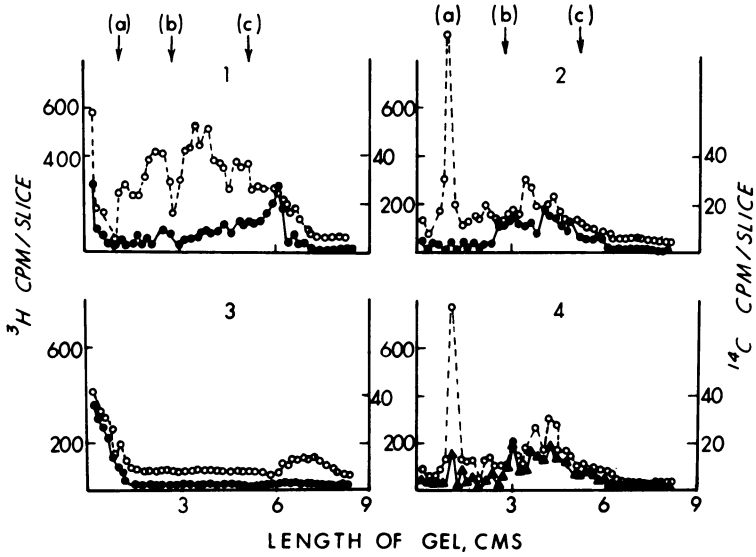

FIG. 2. *Analysis of proteins from subcellular fractions by SDS-polyacrylamide gel electrophoresis. E. coli cultures (CW3747 Pro⁻) were labeled with ^3H-leucine in the presence of proline and ^{14}C-leucine in the presence of the proline analogues. Panels 1, 2, and 3 are cell cytoplasmic, periplasmic, and culture media fractions, respectively, from a culture grown in the presence of 0.2 mM azetidine-2-carboxylate (●). Culture medium was dialyzed exhaustively, lyophilized, and redissolved in 2 ml of 0.5% SDS. Panel 4 is the cell periplasmic fraction from a culture grown in the presence of 0.02 mM 3,4-dehydroproline (▲). All fractions were mixed with the analogous material prepared from cultures grown in the presence of 0.2 mM proline (○). (a), (b) and (c) are markers indicating mobilities of alkaline phosphatase dimer (86,000), monomer (43,000), and large cyanogen bromide fragment (22,000), respectively.*

cant amount of cell protein was localized to the periplasm after the analogue was added. We have been able to localize specifically the alkaline phosphatase made in these cells by using antibodies directed against denatured forms of this enzyme. Table 2 shows that 2% of the periplasmic protein isolated from CW3747 Pro⁻ incubated with the analogue was precipitated by antiphosphatase fragment antibodies. The amount of cytoplasmic protein precipitated was not significantly greater than that found with the unrelated TN antibody preparation. Further information about the proteins precipitated by the antiphosphatase antibodies was obtained from an analysis of the radioactive precipitins in SDS-polyacrylamide gel. In a typical experiment, several precipitates containing protein labeled with ^{14}C-azetidine carboxylate were solubilized, pooled, and subjected to electrophoresis. Figure 3, panel A, shows that more than 60% of the label is in a single peak with a mobility very close to that of authentic alkaline phosphatase subunit. Panel B of the figure shows the anomalous pattern of labeled periplasmic proteins precipitated by the unrelated TN antibodies.

The major protein precipitated by the antiphosphatase fragment antibody has a slightly slower mobility than the normal subunit of the enzyme. By testing ^{14}C-labeled normal subunits precipitated with these antibodies, this difference has been shown not to result from the interaction between antibodies and subunits. The slower mobility could be the result of a distortion in shape brought about by the presence of azetidine carboxylate residues. This is possible even though the gels contain SDS because the normal subunit contains two intact disulfide bonds whereas the azetidine carboxylate polypeptide might have none.

The cytoplasmic protein fractions from exponentially grown cells of CW3747 Pro⁻ labeled with ^{14}C-leucine in the presence of azetidine carboxylate that had been precipitated with the antibody preparations were also examined by SDS-polyacrylamide gel electrophoresis. The profile of radioactive protein from the precipitates with antiphosphatase fragment and anti-TN antibodies were very similar and most of the protein was in a broad band corresponding to molecular weights of 20,000 to 30,000 daltons.

Antimetabolite activity of 4-thiazolidine carboxylate and cis-hydroxyproline. The addition of either 4-thiazolidine carboxylate or

TABLE 2. *Quantitative precipitins with protein from CW3747 Pro⁻ labeled with ^{14}C-leucine in the presence of azetidine-2-carboxylate*[a]

Protein sample	Antibodies	Net counts/min precipitated	Total counts/min added	Percent counts/min precipitated
Periplasm	P'ase fragment	49	2,314	2.1
	P'ase fragment	50	2,410	2.1
	TN	4	2,354	0.2
	P'ase fragment	85	3,375	2.5
	TN	9	3,539	0.3
Cytoplasm	P'ase fragment	50	8,640	0.6
	TN	57	8,357	0.7
	P'ase fragment	57	17,467	0.3
	TN	56	19,396	0.3
	P'ase fragment	111	46,561	0.2
	TN	110	43,340	0.3

[a] Each sample contained, in addition to the cell fractions, 0.1 ml of antibodies, 0.05 ml of M Tris, *p*H 7.4, and was made up to a total volume of 1.0 ml with 0.9% saline. Precipitates were formed after incubation at 4 C for 16 hr, washed three times with cold saline, and collected on membrane filters. P'ase fragment refers to alkaline phosphatase cyanogen bromide fragment (see Materials and Methods). TN antibodies are from rabbits injected with trinitrophenyl-γ-globulin (14).

cis-hydroxyproline to exponentially growing cells of CW3747 initiated linear growth (Fig. 4). The data presented earlier showed that these analogues could not support protein synthesis in the auxotroph. These results showing growth inhibition in the prototroph indicate that the analogues were able to enter the cell. The antimetabolite activity of 4-thiazolidine carboxylate was probably due to feedback inhibition of the proline biosynthetic enzymes. This compound has been reported to be a feedback inhibitor in a cell-free system, and it was also proposed to be incorporated into *E. coli* protein (22), but those experiments were performed with a prototrophic strain. This latter result is illustrative of the problem in interpreting the effects of amino acid analogues under conditions where they must compete with an internal pool of the natural amino acid.

DISCUSSION

The four proline analogues studied in these experiments fall into two general classes. One of these is composed of those analogues recognized by prolyl-transfer ribonucleic acid (RNA) synthetase and capable of being incorporated into polypeptide chains. The other contains analogues that interfere with proline biosynthesis. Table 3 summarizes the data for these analogues. In this regard, these four proline analogues are similar to a variety of amino acid analogues that have been studied extensively (4).

Of the two capable of substituting for proline, because extensive distortion of protein structure, as indicated by the high rate of protein synthesis when it replaces proline, by the qualitatively similar pattern of periplasmic protein revealed by SDS-polyacrylamide gel electrophoresis, and by formation of an active dimeric alkaline phosphatase. The limited amount of this analogue has precluded further study of the substituted enzyme, but the difference in heat stability between the dehydroproline enzyme and the proline enzyme is indicative that substitution has perturbed the structure.

In direct contrast to 3,4-dehydroproline, azetidine carboxylate has a profound effect on bacterial cell metabolism. Not only is overall protein synthesis reduced by 90%, but the SDS-polyacrylamide gel patterns show decreased amounts of high-molecular-weight material. In addition, formation of dimeric alkaline phosphatase is blocked and subunits accumulate. This is not a surprising result considering the model studies reported for this amino acid. X-ray structure studies of Berman et al. showed that the dihedral angle of the peptide bond containing azetidine carboxylate changed some 16° compared to proline (2). Current studies on the amino acid sequence of *E. coli* alkaline phosphatase show that one-third of the prolyl residues are clustered in a span representing only 10% of the entire chain. A cluster composed of azetidine carboxylate would be expected to have a significant effect on the folding of the polypeptide.

We detected alkaline phosphatase subunits containing ^{14}C-azetidine carboxylate only in

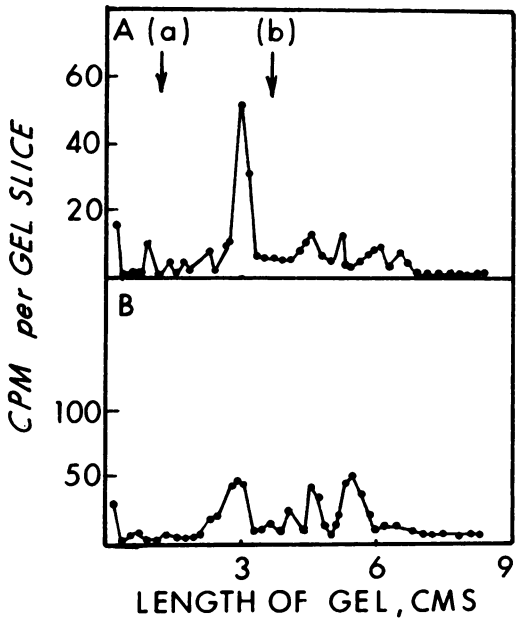

FIG. 3. *SDS-polyacrylamide gel analysis of protein precipitated by antiphosphatase fragment antibodies. Samples of the periplasmic fractions obtained from cells incubated with ^{14}C-azetidine-2-carboxylate (A) were mixed with serum containing antiphosphatase fragment antibodies. For the "control" (B), periplasmic fractions were obtained from CW3747 Pro$^-$ cells labeled with ^{14}C-leucine in the presence of azetidine-2-carboxylate and added to a preparation of unrelated rabbit antibodies (see Table 2 and Materials and Methods). Several of the precipitates were dissolved in a total of 0.2 ml containing 30% sucrose, 10 mM sodium phosphate (pH 7.0), and 0.2% SDS and subjected to acrylamide gel electrophoresis. A total of 340 counts/min were applied to A, and 500 counts/min were used for B. (a) and (b) are markers as described for Fig. 2.*

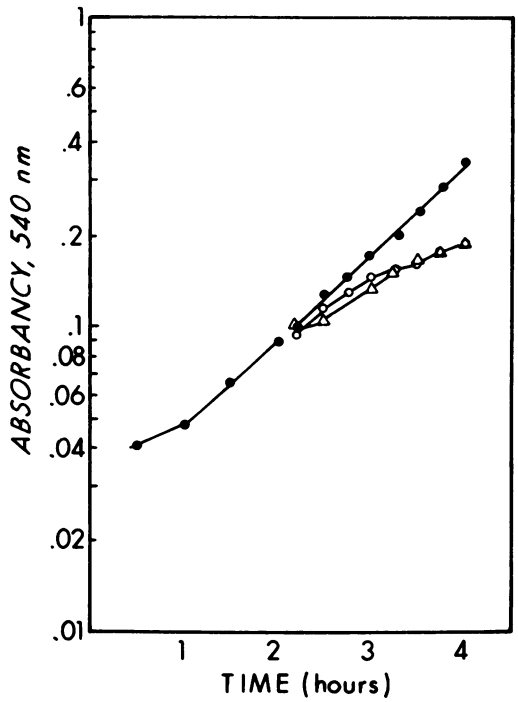

FIG. 4. *Antimetabolite activity of 4-thiazolidine carboxylate and cis-hydroxyproline. Exponentially growing E. coli (CW3747) were divided into three flasks into which 1.5 mM 4-thiazolidine carboxylate (Δ), 1.5 mM cis-hydroxyproline (O), or no amino acid (●) was added. Samples were removed at the indicated times and the absorbance at 540 nm was recorded.*

the periplasmic fraction. Thus, transport of the subunits can proceed even though the chain has become distorted. These results are similar to the previous experiments with two histidine analogues (18, 19). Our conclusion from all of the experiments with amino acid analogues is that structural integrity of the alkaline phosphatase polypeptide is not a crucial determinant in the transport process. Suzuki and Garen have evidence that the protein may not even have to be intact for secretion, for they observed secretion of an amino-terminal fragment from the cytoplasm of an alkaline phosphatase amber mutant (21; Garen, *personal communication*).

If the information for transport of a polypeptide does not reside in the secondary structure of the protein itself, what alternatives remain for selective localization? We propose two possible models: (i) a sequence of amino acids at the amino-terminal portion of the polypeptide is required for transport which might be facilitated by carrier proteins or lipids; or (ii) a sequence of nucleotides in messenger RNA (mRNA) provides for localization of the mRNA to a specific site of synthesis on the plasma membrane. *E. coli* alkaline phosphatase has been reported to contain variable amino-terminal sequences (21). There is evidence that mRNA molecules, particularly those found in bacteriophages, have additional base sequences that are not used for translation of polypeptides (3). In animal cells some mRNA molecules transported from the nucleus contain a stretch of poly(A) residues (8), and such cells have been shown to contain mRNA and ribosomes associated with membranes (10). In mammalian tissues that actively secrete proteins, growing polypeptide chains of these proteins are "directed" into the interior of microsomal fractions, possibly indicative of a "vectorial discharge" of the protein from the polyribosome into secretory vesi-

TABLE 3. *Summary of effects of proline analogues*

Class	Analogue	Antimetabolite	Effect on protein synthesis	Nature of alkaline phosphatase formed
1	4-Thiazolidine carboxylate	+	Stops protein synthesis	None formed
1	*cis*-Hydroxyproline	+	Stops protein synthesis	None formed
2	Azetidine-2-carboxylate	+	Allows for synthesis at 10% that of proline	Subunits
2	3,4-Dehydroproline	+	Allows for synthesis at 80% that of proline	Active enzyme

cles (9).

Although there have been reports that bacterial ribosomes are found attached to cell membranes (20), it will be necessary to show a correlation between the appearance of membrane-bound polyribosomes and increased amounts of periplasmic proteins to corroborate this model. In a recent report, synthesis of an extracellular alkaline phosphatase by *Micrococcus* was found more sensitive to certain inhibitors of protein synthesis than was general cell protein formation (7). This result was interpreted to support a site-specific model for biosynthesis of a secreted protein.

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