Alkaline Phosphatase Subunits and Their Dimerization In Vivo

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A pool of alkaline phosphatase subunits has been found in cells of *Escherichia coli* which are actively synthesizing the enzyme. The radioactive subunits from pulse-labeled cells were specifically recognized by their capacity to produce, upon incubation with Zn^{++} and nonradioactive monomers, radioactive dimers with the characteristics of alkaline phosphatase. The pool of subunits was larger (10 times or more) than the amount expected to be bound to ribosomes and was bound to a rapidly sedimentable fraction from which 60% was released by ribonuclease. In a culture pulse-labeled for one-third (8 sec) of the enzyme synthetic time, the pool of radioactive monomers was 81% of the radioactive enzyme and was totally (98%) in the endoplasm. The size of the pool was increased by decreasing the dimerization rate without affecting protein synthesis. This was achieved by decreasing Zn^{++} in the growth medium. It was found that the cells contained a full complement of monomers, although the level of active enzyme was low. A process subsequent to the release of the monomers from the ribosomes was found to be limiting the formation of the finished enzyme. This process affects the level of the pool of monomers independently from their synthesis.

Polypeptidic chains formed on the ribosomes probably undergo a series of intermediary steps in the process of assuming their final structure as active proteins. Which of these steps may occur while the chain is still attached to the ribosomes is a matter for investigation. The question regarding enzymes containing more than one polypeptide subunit is whether the condensation of the polypeptides occurs before or after the release of the individual chains from the amino acid assembly system.

Alkaline phosphatase of *Escherichia coli* consists of two identical polypeptidic subunits (10) and an average of 3 Zn atoms is necessary for the activity of the dimer molecule (9). Previous reports (7; W. A. Warren and D. A. Goldthwaite, Federation Proc., p. 144, 1961) of active alkaline phosphatase associated with ribosomes suggest that dimerization may take place before release from the ribosomes. However, by breaking open cells actively producing the enzyme, there is a high probability of creating artificial binding of enzyme or dimerization of subunits on the ribosomes. On the other hand, a measurable amount of activatable material has never been found in the cell extracts. Therefore, if a pool of monomers exists free from the ribosomes, it may be difficult to recognize because it is ex-

pected to represent a very small fraction of the total enzyme present in the cells. The active enzyme is accumulated in the space between cell membrane and cell wall (periplasm) in vivo (5); therefore, it is possible to eliminate the bulk of the finished dimers by damaging the cell wall [with lysozyme-ethylenediaminetetraacetic acid (EDTA) treatment] (4) before breaking the cells. To demonstrate the existence of monomers and to measure their pool size, it is also necessary to recognize them among the bulk of the other proteins. Therefore, in our experiments, we relied on the conditions specifically leading to dimerization. These conditions are: dependence upon monomers and upon Zn^{++} concentration. All materials which require such specific conditions to produce finished enzyme are considered to be monomers.

In this study, we present evidence for the existence of a pool of monomers released from the ribosomes.

MATERIALS AND METHODS

Bacterial strains. Two strains of *E. coli* K were used: K-10 (originally from L. Cavalli), an Hfr prototroph, and W3747 (originally from the F'13 of Y. Hirota), an F' methionine auxotroph carrying an extrachromosomal segment which includes the phos-

phatase gene and one of the regulatory genes (R_1) of this system. Therefore, W3747 carries several copies of the alkaline phosphatase structural gene. Both strains are repressible and produce the phosphatase only under conditions of inorganic phosphate (P_i) starvation.

Media. Medium "121" had the following composition (g/liter): NaCl, 4.68; KCl, 1.5; NH_4Cl, 1.08; $MgCl_2$, 0.2; Na_2SO_4, 0.35; $CaCl_2$, 29 × 10^{-3}; $FeCl_3$, 0.5 × 10^{-3}; $ZnCl_2$, 0.27 × 10^{-3}; and tris(hydroxymethyl)aminomethane (Tris) "121," 12.00. The pH was adjusted to 7.5 with HCl. After sterilization, P_i (as K_2HPO_4) and glucose were added.

Medium A-P_i, contained (per liter): sodium citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; KCl, 0.15 g; Tris "121," 12 g. The pH was adjusted to 7.4 with HCl. After sterilization, the medium was supplemented with: thiomethyl-β-D-galactoside, 10^{-3} M (final concentration); $ZnCl_2$, 2 × 10^{-6} M; sodium lactate, 6 g/liter; P_i (as KH_2PO_4, 5 × 10^{-4} M); and amino acid mix (20 ml/liter).

The amino acid mix contained 0.5 mg of each of the following amino acids per ml of 0.001 N HCl: glycine, alanine, valine, leucine, isoleucine, serine, threonine, cystine, methionine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, histidine, phenylalanine, tyrosine, tryptophan, and proline. Arginine was excluded because the medium was devised for experiments of incorporation of ^{14}C-arginine. The ^{14}C-arginine (New England Nuclear Corp., Boston, Mass.) had a specific activity of 0.7 mg/mc.

For the "low Zn" medium, spectrochemically pure chemicals (Johnson, Matthey and Co., London, obtained from Jarrell and Ash Co., Mass.) were used. The medium contained (in g/liter): $MgSO_4 \cdot 7H_2O$, 0.1; NH_4Cl, 1.0; and KCl, 1.5. The medium was buffered with 0.1 M Tris Sigma Base and neutralized to pH 7.4 with HCl. After sterilization, 5 g of glucose per liter, "trace elements" to 10^{-7} M, and P_i (as KH_2PO_4) were added. The water was distilled, deionized through an ion-exchanger column (Barnstead Standard), redistilled in glass, and collected and stored in polypropylene (Nalgene) containers. Reagent grade HCl was distilled before use. The sterile concentrated solution of "trace elements" was 10^{-5} M for each of the following (spectrochemically pure) chemicals: $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 5H_2O$, $CoSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$.

From the concentrated solutions of Tris Sigma Base (1 M), glucose (40%), and methionine (1 mg/ml), metal impurities were removed by passage over columns of chelating resins (Bio-Rad AG 50-X8). All glassware and pipettes were soaked for at least 1 week in HNO_3-H_2SO_4 (1:1) (15). Preferably, Nalgene or polycarbonate plastic (Autoclear IEC) containers (not molded in zinc molds) were used after washing overnight in 20% HCl. All solutions and media were sterilized and stored in Nalgene containers. The bacteria were grown in Autoclear IEC tubes. Cotton plugs were sterilized separately. At least six bacterial transfers (at dilutions of 1:10) were necessary to dilute sufficiently the Zn^{++} carried with the inoculum.

The "regular Zn" medium was identical to the above, but was supplemented with 2 × 10^{-6} M $ZnCl_2$.

Zinc analysis. All the analyses were done with an atomic absorption spectrometer (Perkin-Elmer model 303). The bacteria samples for the analyses were suspended in glass double-distilled water and boiled in 5% trichloroacetic acid to extract the zinc.

Derepressed synthesis of alkaline phosphatase. Since *E. coli* strains K-10 and W3747 are repressible, alkaline phosphatase is actively synthesized (16) when P_i becomes limiting (derepression). The cultures growing exponentially in a phosphate-rich medium (8.3 × 10^{-4} M KH_2PO_4) were centrifuged and transferred to the same medium, but without KH_2PO_4. Since the phosphate limitation became increasingly severe, the growth rate decreased gradually while rapid enzyme synthesis started.

Determination of alkaline phosphatase activity. The enzyme activity was measured colorimetrically at 37 C with p-nitrophenylphosphate (NPP) as substrate (17). For very low activities (requiring an incubation of several hours), the following precautions were taken. The cuvettes were acid-cleaned, sterilized, and stoppered. The substrate was dissolved in sterile buffer. Merthiolate (0.1%) and 10^{-2} M NaF were added to the assay. The samples were read against a control without enzyme, incubated and treated in parallel. In this way, it was possible to measure a level of enzyme as low as 10^{-5} units per ml. One unit of enzyme is the amount that gives an increase in absorbancy of 1.0 in 1 min in a 1.0-cm path at 420 nm.

Digestion with Pronase. Some phosphatase samples were treated with Pronase, to which the enzyme protein is resistant (2). Pronase P (B grade, California Corporation for Biochemical Research) was added at a concentration of 100 μg/ml and the digestion mixture was incubated for 30 min at 45 C.

Preparation of phosphatase monomers. The monomers were made from a preparation of purified wild-type enzyme at a concentration of 1 mg/ml in 10^{-2} M Tris buffer containing 10^{-3} M $MgCl_2$. This solution was acidified to pH 2.3 with HCl and kept on ice for at least 1 hr (or until the dimerization treatment). For dimerization to take place, the pH was raised to 7 to 7.4 with 1 M Tris buffer, pH 8.0, and the temperature was raised to 37 C.

Dimerization by "rescue." If the pool of monomers in cells actively making enzyme is very small, it will be diluted further when the cells (10^{10} per ml) are broken open. Thus, the rate of dimerization would be practically zero. In extracts of cells in which the monomers to be measured were labeled with ^{14}C, the rate of dimerization was increased by adding purified unlabeled monomers (rescue) to a final concentration of 100 μg/ml; 10^{-4} M $ZnCl_2$ was also added, and the samples were incubated for 2 hr at 37 C. In one experiment, dimerization was carried out in Nirenberg Standard buffer (NSB) as modified by Levinthal, Signer, and Fetherolf (3; *see* Table 2). After 18 hr of dialysis at 2 C against 10^{-2} M Tris buffer (pH 7.4) containing 10^{-3} M $MgCl_2$ and 10^{-4} M $ZnCl_2$, debris was eliminated by centrifugation. The clear samples were monitored for their content of ^{14}C-labeled trichloroacetic acid-precipitable material and fractionated on diethylaminoethyl (DEAE) cellulose columns. The eluates were monitored for enzyme

activity, which was found in a narrow region of the column (approximately one tenth of its length), and for ^{14}C-labeled trichloroacetic acid-precipitable enzyme. In some of the experiments (Table 2), one sample from the elution tubes containing the highest enzyme activity was precipitated with serum antiphosphatase and another was digested with Pronase and precipitated with trichloroacetic acid. All the trichloroacetic acid precipitates and the serum precipitatives were washed and counted in a gas flow counter along with a sample before precipitation.

Spheroplast formation. The cells were washed in 0.01 M Tris buffer (pH 7.4) containing 0.001 M MgSO$_4$ and resuspended in 20 ml (8.2 × 10^9 cells/ml) of 0.03 M Tris buffer (pH 9) containing 20% sucrose. All manipulations were done at 0 C. Lysozyme (100 μg/ml) was added, followed after 1 min by 10^{-3} M EDTA. The reaction was stopped 15 min later by the addition of 10^{-2} M MgSO$_4$ and 5 μg of deoxyribonuclease per ml. After 5 min, the spheroplast were centrifuged at 27,000 × g for 10 min. The pellet was suspended in 20 ml of NSB buffer (3) in which the spheroplasts lyse by osmotic shock. An equal volume of double-strength NSB was added to the supernatant fluid. Purified, unlabeled monomers (100 μg) were added to samples of the spheroplast lysate and the supernatant fluid for dimerization by rescue.

Preparation of sedimentable fractions. The cultures were chilled by adding ice; the cells were harvested by centrifugation at 2 C and washed once with ice-cold 10^{-2} M Tris containing 10^{-2} M MgSO$_4$, pH 7.4 (Tris-Mg^{++} buffer). The pellet, quickly frozen with acetone and dry ice, was ground with alumina (1 g of pellet and 3 g of alumina) in a mortar at −10 C. During grinding, 0.03 mg of deoxyribonuclease per pellet was added. The alumina paste was suspended in 10 ml of Tris-Mg^{++} buffer. The cell debris and alumina were eliminated by centrifugation at 12,100 × g for 10 min. To separate the sedimentable fraction from the soluble fraction, the extract was layered over 1 ml of 8% sucrose in Tris-Mg^{++} buffer and centrifuged in a Spinco (type 40 rotor) ultracentrifuge for 50 min at 38,000 rev/min. The supernatant fluid (containing most of the soluble proteins and 99.95% of free alkaline phosphatase) was carefully separated from the bottom sucrose layer with a syringe. The sucrose layer was eliminated and the pellet and the walls of the centrifuge tube were rinsed twice with 1 ml of Tris-Mg^{++} buffer cooled at 2 C. The pellet was resuspended in 2 ml of Tris-Mg^{++} buffer and homogenized in a small glass homogenizer. The large aggregates were eliminated by centrifugation for 20 min at 12,100 × g. Centrifugation in a Spinco 40 ultracentrifuge was repeated. The pellet was resuspended in 2 ml of Tris-Mg^{++} buffer, layered on a linear 5 to 20% sucrose gradient, and centrifuged at 24,000 rev/min for 90 min in an SW 25.2 Spinco rotor. The gradient was collected in 25 1-ml fractions after puncturing the bottom of the tube.

RESULTS

Monomers associated with the particulate fraction of cell extracts. The following experiment was designed to determine whether any of the bacterial cell fractions contained material activable by standard procedures of dimerization. Wild-type *E. coli* K-10 was grown in medium "121" and the cells were harvested when active enzyme was being formed. During the incubation (90 min) in P$_i$-free medium, the bacterial mass increased by about 30%. The cell extracts were prepared as described above. The enzyme content in the supernatant (soluble) fraction and in the resuspended pellet (sedimentable fraction) was measured prior to and after a treatment promoting dimerization, i.e., incubation for 30 min at 25 C with 2 × 10^{-4} M ZnCl$_2$. The pelleting of the dimerized particulate fraction through a sucrose layer was repeated to remove residual soluble enzyme and the purified particulate fraction was finally fractionated on a sucrose gradient.

Of the phosphatase activity demonstrable in the unfractionated clear extract, 99.95% was found in the supernatant fraction (Table 1). As expected, no increase in activity after incubation with Zn^{++} could be demonstrated in the highly active supernatant fraction. In the pellet, however, which originally contained only 0.022 enzyme units, the activity increased to 0.224 units after incubation under conditions specifically promoting dimerization. (No increase in activity was found when Zn^{++} was omitted and/or the temperature kept at 0 C.) This suggests that monomers are present when the cell is broken open and that they are associated with the particulate fraction. The distribution of ribonucleic acid (RNA) and of enzyme activity in the fractions lighter than 70S, 70S, and heavier than 70S was determined after fractionation in a sucrose gradient of the purified particulate fraction (Table 1). It was found that 80% of the enzyme was associated with a component larger than the ribosomes. The enzyme activity per milligram of RNA in this fraction was 1.0; in the 70S fraction, it was 0.004. These values indicate that the bulk of RNA is in the 70S fraction, while the bulk of the enzyme is in the heavier fraction. Moreover, a sample of the heavier fraction was digested with ribonuclease and pelleted again. The enzyme activity remaining associated with the portion not degraded by ribonuclease treatment was again measured; 40% was still attached to a rapidly sedimenting fraction. This implied that monomers released from the ribosome accumulated elsewhere prior to dimerization, possibly at the membrane level, as has been previously suggested (8, 14).

Existence of a pool of monomer and its localization with respect to the cell membrane. The experiment described above suggested that monomers were present in the cell extract associated

TABLE 1. *Enzyme activity in different cellular fractions*[a]

Fraction of the clear extract	Enzyme activity (units)[b]	
	Before dimerization	After dimerization
Soluble fraction	52.	52.
Particulate fraction...............	0.022	0.224

Fractionation of particles	Enzyme activity (%)[c]
<70S	10.5
70S	9.5
>70S	80.0
>70S after ribonuclease	40.0

[a] Under our experimental conditions, it is assumed that a cell contains 15,000 ribosomes, of which only 80% are actively synthesizing proteins (1), and that 5% of the active ribosomes are involved in phosphatase synthesis (2). Since each phosphatase subunit is 300 amino acids long in each cell at any given time, the number of ribosomes carrying a finished subunit should be estimated as close to 2. It is known that 1 mole (mol wt $= 4 \times 10^4$) of phosphatase subunits (6×10^{23} subunits) possesses 6×10^{10} activity units (0.66 μg) of phosphatase possess 1 activity unit (17). Assuming that only completed subunit chains can give active dimers, each cell would have no more than 2×10^{-13} activity unit attached to the ribosomal fraction. According to these assumptions and calculations in this experiment, no more than 0.014 activity units should be found associated with the ribosomal fraction in 1 ml of clear extract from 7×10^{10} cells, whereas an increase of 0.200 in activity units after dimerization was found in the particulate fraction. This represents 14-fold more monomers (finished chains) than those expected to be bound to the ribosomes. Dimerization was performed in concentrated extracts corresponding to 2.8×10^{11} cells per ml.

[b] Calculated per ml of the original clear extract; 1 ml of clear extract corresponds to 7×10^{10} cells.

[c] Percentage of the total activity in the particulate fraction prior to sucrose gradient fractionation.

with the particulate fraction and in an amount exceeding that found attached to the ribosomes. This indicated that a step subsequent to any performed by the ribosomes was limiting the formation of active enzyme. It also suggested that such a step may occur at the level of the membrane. The following experiment made use of the spheroplast technique for separating two fractions, outside and inside the membrane, from cells in which the newly formed monomers were labeled preferentially to the dimers. This preferential labeling was obtained by a radioactive

pulse lasting for a time shorter than that required for complete enzyme synthesis.

Cells of strain W3747 (grown overnight in medium A-P$_i$) were incubated in P$_i$-free medium for 50 min to permit phosphatase synthesis by derepression. At this time, the culture was pulse-labeled with ^{14}C-arginine (1 μc/ml) for 8 sec. The pulse was stopped by fast cooling and the cells were harvested immediately thereafter. From data obtained under analogous conditions of derepression, the synthetic time of alkaline phosphatase was estimated (R. Byrne, Ph.D. Thesis, Massachusetts Institute of Technology, 1963) as three times longer than the pulse. The cells were washed twice and converted into spheroplasts by lysozyme-EDTA treatment. As expected, 98% of the active enzyme was released in the supernatant fraction. The spheroplasts were separated by centrifugation and broken open by osmotic shock (spheroplast lysate). Because the presence of intact cells in the spheroplast preparation or the lysis of spheroplasts before their separation from the supernatant fluid would obscure the results, two determinations were made in each experiment as a control at this step: (i) the number of intact cells still able to produce colonies in the spheroplast lysate, and (ii) β-galactosidase activity in the supernatnt fluid. Since it is known that this enzyme is retained inside the spheroplasts (4), the amount of β-galactosidase in the supernatant fluid is a measure of the amount of spheroplast lysis before the osmotic shock. In the experiment summarized in Table 2, the number of

TABLE 2. *Phosphatase production in 8 sec of protein synthesis; partitioning of monomers and dimers between endoplasm and periplasm*

Protoplast fraction	Total proteins[a]	Phosphatase[b]		
		After dimerization[c]	Before dimerization[d]	Δ (monomers)
Supernatant fraction (periplasm)	1,155.0[e]	50.6	46.0	4.6
Lysate (endoplasm)	11,400.0	241.0	8.0	233.0

[a] Counts per min precipitable by trichloroacetic acid.

[b] Counts per min precipitable by specific serum after DEAE chromatographic fractionation.

[c] Includes dimers + monomers.

[d] Dimers only.

[e] The figures are counts per min and per 8.2×10^9 cells.

intact cells still able to produce colonies in the spheroplast lysate were found to be small enough to be ignored (0.1 intact cells per 100 of the original population), whereas, determination of β-galactosidase activity in the supernatant fluid indicated that 16% of spheroplast lysis had occurred prior to the osmotic shock. In accordance with these results, an appropriate correction was introduced in the calculation of the pertinent values.

The total amount of ^{14}C-labeled proteins formed during the pulse was determined in the two fractions (supernatant and spheroplast lysate) by measuring the radioactivity of the trichloroacetic acid-precipitable material in small samples of the fractions. The number of ^{14}C-labeled monomers among the newly formed proteins was determined by measuring the radioactivity of the "rescued" purified enzyme resulting after their dimerization in vitro (see above). That the "rescued" ^{14}C-labeled material is, in fact, finished enzyme was proven by three independent criteria: (i) its position on DEAE columns, (ii) its stability to Pronase treatment, and (iii) its specific precipitation by serum antiphosphatase. (It is known that, under these conditions, the phosphatase dimers are essentially the only protein not hydrolyzable by Pronase.) Furthermore, the number of counts precipitable with serum antiphosphatase, from a sample to which unlabeled dimers instead of unlabeled monomers were added at the "rescue" step, was low and comparable to the basal level obtained in the absence of any addition. This also verified the assumption that dimerization was the factor responsible for serum-precipitable radioactivity above the basal level.

This experiment is summarized in Table 2, in which the serum-precipitable radioactivity is referred to as enzyme counts. Table 2 shows that 82% of the serum-precipitable radioactivity was "rescued" by dimerization; 98% was found in the endoplasm and 2% in the periplasm. The total amount of ^{14}C-phosphatase was 2.3% of the total ^{14}C-proteins formed during the pulse. In fully derepressed cells of E. coli W3747, phosphatase represents 15% of the total proteins, but cells used for the experiment summarized in Table 2 were harvested when the cells were only one-third derepressed.

Zn^{++} *concentration in the culture medium and rate of dimerization.* The rate of dimerization in vitro is Zn^{++}-dependent (11, 12), and it was surmised that a similar dependence would be demonstrable in vivo. Each dimer molecule contains an average of 3 atoms of zinc, necessary for enzymatic activity, but more zinc can be bound in

vitro (M. J. Schlesinger and G. A. Reynolds, *personal communication*). It is conceivable that only a few proteins in the cell are dependent on such a high level of zinc. Thus, it was anticipated that by lowering the level of Zn^{++} in the culture medium, it would be possible to interfere selectively with the dimerization rate of alkaline phosphatase and to grow cells which could produce regular amounts of monomers but which would be impaired in their ability to form dimers. To verify these predictions, several experiments were performed.

Because it is practically impossible to decrease the Zn^{++} concentration to less than one-tenth of the usual amount (2×10^{-6} M), the "low Zn" medium contained Zn^{++} at about 10^{-7} M. A culture of E. coli K-10, maintained in "low Zn" medium for 12 transfers, was inoculated into "low Zn" and "regular Zn" media, each containing the same limiting amount of P_i (1.33×10^{-4} M K_2HPO_4). The two cultures were grown overnight to the stationary phase to fulfill the conditions for maximal phosphatase derepression. At this time, the enzyme level was measured in a sample of cells from the two cultures. Samples of each culture were further incubated for 6 hr at 37 C, the "low Zn" culture under two conditions of Zn^{++} concentration: (i) unchanged, and (ii) raised 10-fold to the regular level. At the end of this incubation, the enzyme level was measured again. The bulk of the two cultures was centrifuged and the zinc content was measured in the supernatant fluid and in the pellet. The final yield of bacteria from the two cultures was practically the same, whereas the enzyme level differed by a factor of 3, the "low Zn" cells possessing 34% of the activity contained in the "regular Zn" cells (Table 3). However, after the additional 6 hr of incubation, during which no further growth was observed in any culture, the

TABLE 3. *Effect of Zn*[++] *in the medium on the level of active enzyme*[a]

Incubation of the stationary phase culture	Culture medium		
	"Low Zn"		"Regular Zn"
	No addition	Zn added	
Zero-time	3.77	3.76	11.0
After 6 hr	3.77	8.50	12.3

[a] Strain K-10 grown under P_i starvation until the stationary phase was reached in "regular Zn" or "low Zn" media. The figures are enzyme units in cell extracts per optical density unit of the culture.

sample from "low Zn," in which Zn^{++} was raised, showed an enzyme increase to a final level at 65% of that reached by the "regular Zn" culture.

By comparing the Zn^{++} content of the supernatant fractions and the pellets of the two cultures (Table 4), it was clear that Zn^{++} was concentrated into the cells against the gradient. The level of Zn^{++} in the water space of cells grown in "regular Zn" medium was about 3×10^{-4} M, which is of the same order of magnitude as the optimum required for dimerization in vitro. In cells grown in "low Zn" medium, this concentration was lower by a factor of 2.9. It was concluded that a Zn^{++} concentration of 2×10^{-7} M, which appeared to be sufficient for regular growth, impaired conspicuously the production of active enzyme.

The obvious interpretation of these results (Table 3) is that protein synthesis, including monomer synthesis, was not affected by these variations in Zn^{++} concentration. The rate of dimerization, however, appeared to be largely reduced during growth in "low Zn" medium and monomers appeared to accumulate. An amount of monomers equivalent to that in "regular Zn" medium should be produced, but they are probably unstable, as was suggested by the experiments in vitro (13). This instability accounts for the low recovery of dimerized enzyme obtained in this experiment.

In a final experiment, the amount of monomers produced during a ^{14}C-pulse was measured. Parallel cultures of *E. coli* K-10 were grown in "low Zn" and "regular Zn." After 2 hr of P_i starvation, the two cultures reached an optical density of

TABLE 4. Zn^{++} concentration in different parts of the culture[a]

Part of culture	Medium	
	"Regular Zn"	"Low Zn"
Bacterial yield (cultural OD).................	0.762	0.745
Zn in the supernatant fraction (μg/liter).....	122.0	12.3
Zn in the pellet (μg/liter).................	15.9	5.4
Zn in cellular water space (μg/liter)[b].......	16,900.0	5,750.0
Zn concn factor.........	×138	×465

[a] Same stationary phase cultures of K-10 used in Table 3.

[b] Pellet from 1 liter of culture at 0.750 OD weighs 1,200 mg (wet weight) and 260 mg (dry weight). The water space pertaining to the cells is 0.940 ml.

0.500 and were actively producing phosphatase. At this time, an 11-min pulse of ^{14}C-arginine (1 μc/ml) was given. The labeling was stopped by quick-cooling; the cells were harvested and opened with a French Press. The ^{14}C-monomers were "rescued" by dimerization in the presence of 100 μg of unlabeled wild-type monomers per ml and 2×10^{-4} M $ZnCl_2$. After dimerization, the enzyme was purified from the crude extract by fractionation on a DEAE column. The band of active enzyme was further purified by fractionation in a 5 to 20% linear sucrose gradient (Table 5). In the control, cells grown in "regular Zn" medium, 5.8% of the total ^{14}C-proteins was present as dimers and 0.8% as free monomers. The cells grown in "low Zn" possessed 40% of the control enzymatic activity, but they contained almost the full complement of ^{14}C-monomers (95%). The supernatant fractions of these cultures were analyzed in the same manner. The peak of enzyme activity in the sucrose gradient did not correspond to the peak of radioactive material, indicating that the supernatant fluids of these cultures did not show any measurable radioactive dimers after reaction with cold monomers. We concluded that, in 11 min of growth, the "low Zn" cells synthesized the expected amount of monomers, of which 55% were dimerized and 45% were found as free monomers inside the cells.

It was therefore confirmed that, in the "low Zn" conditions of these experiments (2×10^{-7} M Zn^{++}), only the dimerization was impaired, whereas synthesis of monomers proceeded normally. However, in cultures reaching the stationary phase of growth, the free monomers were partially lost or irreversibly denatured.

DISCUSSION

In the first experiment, the cells were harvested when they were actively producing alkaline phosphatase. The sedimentable fraction was obtained from the cell extract in the classical way used for ribosome separation. This fraction showed almost no phosphatase activity (0.022 enzyme units per unit of cells), but activity was produced (0.224 units) after a short incubation in the presence of Zn^{++}. Since no activity was observed when Zn^{++} was omitted and the temperature kept low, and since it is known that dimerization is zinc and temperature dependent, the indication is strong that the observed increase in activity was due to dimerization of monomers. Because this increase represents only 0.4% of the total activity in the cell, it is not surprising that this increase was unnoticed after similar incubation of the unfractionated crude extract. In the second experiment, another culture which

TABLE 5. *Monomers and dimers produced in 11 min of protein synthesis[a]*

Phosphatase	"Low Zn" medium			"Regular Zn" medium		
	Total proteins, 648,000[b]			Total proteins, 445,000[b]		
	After dimerization[c]	Before dimerization[c]	Δ (monomers)	After dimerization[c]	Before dimerization[c]	Δ (monomers)
Enzyme[d].............	36,000	20,000	16,000	26,000	22,500	3,500
Enzyme (as percentage of ^{14}C-proteins)......	5.5	3.1	2.48	5.8	5.1	0.8[e]

[a] *E. coli* K-10 strain grown for 2 hr in P_i starvation to OD = 0.500 in both cultures. Enzyme activity, 1.7 units per OD in "regular Zn" and 0.7 in "low Zn."

[b] Counts per min and per 10^{10} cells.

[c] Incubation with 2×10^4 M $ZnCl_2$ in the presence (after) or in the absence (before) of cold monomers.

[d] Purified ^{14}C-enzyme; counts per min per 10^{10} cells.

[e] This value is 13.5% of the total enzyme (5.8) accumulated in 11 min. From the data in Table 1 (in which the subunits represent 0.4% of the amount accumulated in 90 min of derepression), it is possible to calculate the per cent of enzyme expected in the conditions of this experiment. Assuming a doubling time of 240 min, the expected value is 13.3%.

was in a phase of active production of enzyme was pulse-labeled with ^{14}C-arginine. After 8 sec, the cells were harvested and spheroplasts were made to eliminate the bulk of active enzyme. The endoplasm was obtained by osmotic shock of the washed spheroplast and, without further fractionation, was examined for the presence of radioactive monomers produced during the pulse. Here, again, the experimental approach was the same, i.e., determination of active enzyme before and after an incubation treatment which should promote dimerization. However, the enzyme was further separated by DEAE fractionation and measured by radioactivity precipitable with an immune serum specific for the dimers (and not for the monomers). Moreover, the incubation conditions were different. The increase in dimers precipitable by serum was obtained upon incubation in the presence of Zn^{++} and also in the presence of the externally added unlabeled monomers (rescue). Without the addition of monomers or with the addition of dimers, no increase in labeled material precipitable by serum was observed. The concentration of monomers had to be raised under the conditions realized in the second experiment, but not in the first, because of the properties of the reaction of dimerization. Since it is a bimolecular reaction, no spontaneous dimerization is observed unless the concentration of the subunits is high. This condition was met by a high concentration of cells, as in the first experiment (2.8×10^{11} cells per ml), or by the addition of monomers at a high concentration (rescue), as in the second experiment.

Given the mechanism of protein synthesis, it is expected that, if monomers are present in the cell, the ribosomes involved in phosphatase synthesis would bear only monomers and not dimers. Therefore, to understand the relationship between polypeptide chain synthesis and dimerization, we had to determine whether there is an excess of monomers above the amount bound to the ribosomes. In the first experiment, the sedimentable fraction of the cells after dimerization treatment was fractionated further through a sucrose gradient, and the distribution of enzyme activity among the different components was determined. Even assuming that the fraction lighter than 70S resulted entirely from ribosomal degradation and that the portion hydrolyzed away by ribonuclease from the fraction heavier than 70S was entirely due to polysomes, about 40% of the activity still remained associated with particles heavier than 70S, which apparently are not polysomes. It could be argued that ribosomes may be trapped in the heavier fraction, not accessible by ribonuclease. However, considering the ratio of enzyme to RNA of the 70S fraction, the amount of RNA found in the heavy fraction was 200 times less than expected on the basis of the amount of enzyme present in it. Furthermore, the calculations given in footnote *a* to Table 1 offer an additional indication that the monomers found were in excess (14 times) of what could be expected to be attached to the ribosomes. Thus, we conclude that only a fraction of the monomers found experimentally are bound to the ribosomes.

Directly measured data on monomers attached to ribosomes are not available from the second experiment. However, 81% of the total dimerizable counts were monomers, and 98% of the monomers were found in the endoplasm. From the results of the two experiments, it may thus be concluded that monomers accumulate somewhere in the cell after leaving the ribosomes.

It is possible to change the level of this reservoir by decreasing the flow from the finished subunits to the dimers. This can be done by realizing the condition of Zn^{++} deficiency which affects the rate of dimerization without affecting the rate of synthesis of the proteins. It was found that in the cells of the "low Zn" culture, which reached the stationary phase because of P_i limitation, the enzyme level is low (10 to 30% of that from the "regular Zn" culture). However, if Zn^{++} concentration is raised, the enzyme activity increases to 69% of that found in the regular zinc culture, although the cell mass does not change. By contrast, in the "regular Zn" culture, the enzyme level rises only 10% upon further incubation. Therefore, material is accumulated which may be activated by Zn^{++}. Furthermore, the data in Table 5 show that this activation also depends on the concentration of monomers; if the "rescue" step is omitted, dimerization does not occur. Thus, the last two experiments together indicate that the rate of dimerization may be slowed by limiting the Zn^{++} supply without affecting the rate of monomer synthesis. It is concluded that the two processes are mutually independent.

Some useful indications about zinc uptake in the cellular pool and the function of it have been obtained in the course of experiments in which Zn^{65} was used (*unpublished results*). The results indicate that the cells are able to concentrate zinc against the gradient and only 50% of this zinc may be considered as an exchangeable pool. The remainder is irreversibly fixed. An indication that a substantial part of it is bound to alkaline phosphatase is obtained by determining the variation of the zinc level in the cells which are switched from a condition of derepression to repression. When maximal repression is reached, the zinc content stabilizes at a level which is 20% lower than that at maximal derepression.

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