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FACTORS AFFECTING THE FORMATION OF ALKALINE PHOSPHATASE ISOZYMES IN *ESCHERICHIA COLI* K-12

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SUMMARY Physiological and genetic factors affecting the formation of isozymes of alkaline phosphatase in *Escherichia coli* K-12 were studied.

Our results are compatible with the hypothesis proposed by Schlesinger and his co-workers (Schlesinger et al., 1975) that the multiple forms of the enzyme are produced by converting a newly synthesized one (the least negatively charged one) into less negatively charged forms. Neither energy source nor *de novo* synthesis of the enzyme was necessary for the conversion. It is also confirmed that the conversion is effectively inhibited by externally added arginine (Piggott et al., 1972) but only partially by canavanine (arginine analog) or casamino acids.

We isolated a mutant strain which did not form isozyme(s), if any, under the condition in which the wild type strain formed isozymes. The mutation(s) was proved to be mapped in the locus (or loci) other than alkaline phosphatase structure gene in the *E. coli* genetic map. We tentatively proposed to designate this the *iap* gene(s), an abbreviation for *i*sozyme of *a*lkaline *p*hosphatase, which plays a role in isozyme formation.

INTRODUCTION

The enzymatically active alkaline phosphatase (EC 3.1.3.1) of *E. coli* K-12 is a dimer composed of two identical polypeptides, which is coded for by a single structure gene (Garen and Garen, 1963; Rothman and Byrne, 1963). Nevertheless, several (mainly three) distinct and enzymatically active forms are found when the purified enzyme is analysed by starch gel or polyacrylamide gel electrophoresis (Garen and Levinthal, 1960; Signer et al., 1961; Schlesinger and Andersen, 1968; Piggott et al., 1972). These isozymes are not the results of an artifact during isolation, purification and analysis, nor the results of multiple aggregation of subunits, but are formed as the results of cytoplasmic factors (Signer, 1963. Ph. D. Thesis in Massachusetts Institute of Technology, cited from Schlesinger and Andersen, 1968).

Mainly two different explanations have been proposed to explain the formation of these multiple forms of enzyme. The one is a modification of the enzyme molecules after synthesis either by association with sugar moie-

ties (Reid and Wilson, 1970) or removal of amino acids from polypeptides (Suzuki and Garen, 1969; Natori and Garen, 1970; Schlesinger et al., 1975). The other is the result of an ambiguous translation of a single genetic message, caused by mutational changes in ribosomal proteins (Piggott et al., 1972). The evidences supporting the former hypothesis have been presented, that is, the amino-terminal arginine residue in the least negatively charged form is removed resulting in conversion into more negatively charged forms, in which the resulting amino-terminal is threonine residue (Kelley et al., 1973; Schlesinger et al., 1975).

In this paper, we have investigated both physiological and genetic factors affecting the isozyme formation and present evidence that the chromosomal gene(s) in *E. coli* is also responsible for the conversion of newly synthesized enzyme.

MATERIALS AND METHODS

1. *Bacterial strains*

E. coli K-12 strains used in this study and their sources were described in Table 1.

TABLE 1. *Escherichia coli K-12 strains used in this study*

Strain	Genotype[a]	Source
AN234	Hfr C, *phoT*[b], *iap*[c]	After treatment C90 with NG
C80	Hfr C, *phoR*	F. Rothman
C90	Hfr C, *phoT*[b]	N. Otsuji
K10	Hfr C	F. Rothman
LC607	F$^-$, *leu*, *proC*, *purE*, *trp*, *lys*, *metE*, *thi*, *ara*, *lacZ*, *xyl*, *rpsL* (*strA*), *tonA*, *tsx*	K. Matsubara
U24R3Ua	Hfr C, *phoA*$^{+d}$	F. Rothman
W3747	F'13/*met*	A. Garen

[a] All gene symbols are from Bachmann et al. (1976), except where otherwise noted.

[b] *phoT* is originally designated as R2b$^-$ (Garen and Otsuji, 1964; Willsky et al., 1973).

[c] A mutation, newly isolated by us, which is unable to form isozyme of alkaline phosphatase.

[d] *phoA*$^+$ gene of U24R3Ua is a reverse mutation of *phoA*$^-$ (U24), and produces alkaline phosphatase moving faster than the wild type enzyme on electrophoresis.

2. *Chemicals*

Trizma base [tris(hydroxymethyl)aminomethane] and Fast Blue B salt (tetrazotized *o*-dianisidine) were purchased from Sigma Chem. Co. Acrylamide, bisacrylamide (*N,N'*-methylenebisacrylamide), TEMED (*N,N,N',N'*-tetramethylethylenediamine), disodium α-naphthyl acid phosphate and disodium *p*-nitrophenyl phosphate were purchased from Wako Pure Chem. Co. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was purchased from Nakarai Chem. Co.

3. *Buffer*

Tris-Mg^{++} buffer used in this study was 0.01 M Tris-HCl, pH 7.2–0.001 M $MgSO_4$.

4. *Culture media*

T salts medium contained 1.2×10^{-1} M Tris-HCl, pH 7.2, 8×10^{-2} M NaCl, 2×10^{-2} M KCl, 2×10^{-2} M NH_4Cl, 10^{-3} M $MgCl_2$, 2.5×10^{-3} M Na_2SO_4, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$ and 2×10^{-6} M $FeCl_3$. TG medium was T salts medium supplemented with glucose (0.2%) and TGC medium was TG medium supplemented with casamino acids (0.2%), unless otherwise stated. These media were also supplemented with either excess (6.4×10^{-4} M) or limited (6.4×10^{-5} M) KH_2PO_4.

T broth contained 10 g Bacto-tryptone (Difco), 5 g NaCl per liter. LB broth contained 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl per liter and 1 N NaOH was added to adjust pH 7.2. Broth and TG medium plate contained 1.3% agar.

5. *Preparation of spheroplast*

The EDTA-lysozyme procedure of Morris et al. (1974) was followed with slight modification. Bacterial cells were harvested by centrifugation and washed in ice-cold 0.1 M Tris-HCl, pH 8.0. The cell pellets were resuspended in one-twentieth of the original volume of 0.03 M Tris-HCl, pH 9.0 containing 0.5 M sucrose. To the cell suspension was added one-hundredth volume of lysozyme (10 mg/ml), and two min later one-hundredth volume of EDTA (0.1 M) was added. After 15 min at 4C, one-hundredth volume of $MgSO_4$ (1 M) was added

to the mixture. After a further 20 min at 4C, the cell (spheroplast) suspension was centrifuged at 23,000×*g* for 15 min and the supernatant was dialyzed against Tris-Mg^{++} buffer (alkaline phosphatase was released into supernatant of spheroplast).

6. *Transduction by bacteriophage P1*

The procedures of preparation of P1 phage lysate and transduction have been described previously (Nakata et al., 1971). The P1 phage used in this experiment was a virulent mutant strain (P1*vir*) obtained from J. Tomizawa.

7. *Enzyme assay*

Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate (10^{-3} M in 1 M Tris-HCl, pH 8.0) as a substrate at 30C. The reaction was stopped with one-fifth volume of 0.5 M Na_2HPO_4 and the developed color was measured at 410 nm by Hitachi UV-VIS spectrophotometer model 139.

8. *Mutagenesis by NG*

A culture of strain C90 in LB broth was treated with 100 μg/ml of NG during early logarithmic phase of growth at 37C for 60 min with shaking (Glover, 1968). After 60 min, the cells were washed twice and resuspended into T salts medium, and then an aliquot of the suspension was plated on T broth plate. A single colony was picked and suspended into 1 ml of T broth, and then incubated at 37C overnight. After the cells grew, an equal volume of 80% sterile glycerol was added to each culture, mixed well and kept at −15C freezer (mutagenized stock culture).

To examine the isozyme pattern of alkaline phosphatase, an aliquot of mutagenized stock culture was inoculated into 5 ml TG medium supplemented with excess phosphate and casamino acids (0.002% instead of 0.2%), and then incubated at 37C with shaking. After overnight incubation, the cells were harvested and resuspended into 2 ml Tris-Mg^{++} buffer. To the cell suspension was added one drop of toluene, agitated vigorously and then it was heated at 90C for 15 to 20 min. An aliquot of the supernatant was applied on gel electrophoresis.

9. *Gel electrophoresis*

Gel electrophoresis was carried out according to the method of Maizel (1971). Slab type polyacrylamide gel was composed of 10 ml of acrylamide-bisacrylamide solution (30: 08), 10 ml of 1.5 M Tris-HCl, pH 8.8, 0.02 ml of TEMED, 0.24 ml of $(NH_4)_2SO_4$ (10% in H_2O) and 20 ml of H_2O, and spacer gel was composed of 1.33 ml of acrylamide-bisacrylamide solution (30: 08), 1.25 ml of 0.5 M Tris-HCl, pH 6.8, 0.005 ml of TEMED, 0.1 ml of $(NH_4)_2SO_4$ (10% in H_2O) and then made up a total volume of 10 ml with H_2O. The electrode buffer contained 0.6 g Trizma base and 2.88 g glycine in a liter of H_2O. Electrophoresis was carried out at 300 v constant voltage for 3 hr at room temperature.

After electrophoresis, the gel was stained with a mixture of 12 ml of α-naphthyl acid phosphate (2 mg/ml in 1 M Tris-HCl, pH 8.0) and 4 ml of Fast Blue B salt (20 mg/ml in H_2O) and 100 ml of H_2O. When the color developed, the gel was washed in tap water and kept in 7% (v/v) acetic acid, and then dried on a filter paper in vacuo.

RESULTS

1. *DEAE-cellulose column chromatography*

A culture of strain W3747 was labeled with ^{14}C-lysine for 60 min after 12 hr starvation of phosphate (derepression of alkaline phosphatase). Alkaline phosphatase was isolated with EDTA-lysozyme procedure, and chromatographed on DEAE-cellulose column. The peak of enzyme activity was found to be eluted at a higher concentration of NaCl than that of radioactivity (Fig. 1). An aliquot of both peaks was mixed and applied on sucrose density gradient centrifugation (5 to 20%). Centrifugation was carried out on SW39 rotor of Spinco at 35,000 rpm for 15 hr. But the peak of enzyme activity could not be separated from that of radioactivity.

Prolonged labeling of the alkaline phosphatase with radioactive amino acid resulted in coincidence of both peaks on DEAE-cellulose column chromatography. It was likely that in the presence of added amino acids as a lysine coolant, one form of alkaline phosphatase (the least negatively charged isozyme) was labeled rapidly, and then the enzyme was labeled equally during further incubation.

2. *Gel electrophoresis*

Alkaline phosphatase of strain K10 syn-

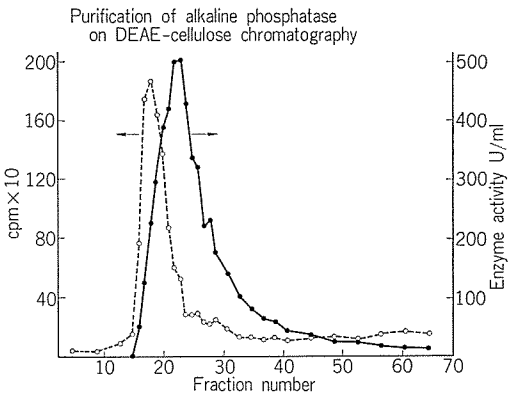

FIGURE 1. A culture of W3747 in TG medium supplemented with excess phosphate was harvested by centrifugation during logarithmic phase of growth and resuspended into TG medium without added phosphate. Incubation was continued at 37C with shaking (derepression of alkaline phosphatase). Twelve hours after deprivation of phosphate, a mixture of proline, arginine, leucine (at a final concentration of 62.5 μg each per ml) and threonine and glutamic acid (at a final concentration of 15.2 μg each per ml) was added. After 10 min, ^{14}C-lysine was added to the culture at a final concentration of 0.1 μCi/ml. After 60 min labeling, the culture was harvested by centrifugation and the cells were converted to spheroplasts with EDTA-lysozyme procedure. Supernatant of spheroplasts was dialysed against Tris-Mg^{++} buffer. Dialysate was treated with DNase (at a final concentration of 10 μg/ml) and RNase (at a final concentration of 5 μg/ml) at 37C for 30 min and then heated at 90C for 10 min. Aggregation of denatured proteins was removed by centrifugation and the supernatant was dialysed against Tris-Mg^{++} buffer. Final dialysate was applied on DEAE-cellulose column chromatography. Elution was carried out with linear gradient of NaCl (0 to 0.4 M) in Tris-Mg^{++} buffer. ——, enzyme activity; - - -, radioactivity.

thesized in TGC medium supplemented with limited phosphate was partially purified as described in the legend to Fig. 1, and applied on gel electrophoresis. Three distinct enzymatically active bands were observed. The same preparation of the enzyme was then applied on DEAE-cellulose column chromatography. A broad enzyme peak was obtained after elution with very shallow gradient of NaCl (0.05 to 0.25 M), and then several fractions were analyzed on gel electrophoresis. Three enzymatically active bands were observed in each fraction. But, in the front fractions (eluted at lower concentration of NaCl), the rapidly moving band (the most negatively charged enzyme) was apparently stained faintly as compared with the slowly moving one, and the band moving last (the least negatively charged one) was stained most deeply. In the later fractions the rapidly moving band was stained deeper than the slowly moving band.

Of the discordance of radioactivity and enzyme activity on DEAE-cellulose column chromatography, described in the previous section, it is very likely that the highly labeled enzyme eluted at lower concentration of NaCl is the least negatively charged form and the bulk enzyme is more negatively charged and eluted at higher concentrations of NaCl.

Since these three isozymes are thought to correspond to those reported by Schlesinger and Andersen (1968), we, hereafter, call the most slowly moving isozyme on electrophoresis or the least negatively charged one isozyme 1, the most negatively charged one isozyme 3 and the isozyme in between isozyme 2, according to their designation.

The fact that, when the enzyme was synthesized in TGC medium, the isozyme 1 (or all three isozymes) was synthesized predominantly, and in TG medium without supplemented amino acids (or casamino acids), the isozyme 3 was produced predominantly indicated that, in the presence of added amino acids, isozyme 1 was synthesized, therefore, rapidly labeled with radioactive amino acid, and was eluted at a lower concentration of NaCl on DEAE-cellulose column chromatography, as mentioned in the previous section.

3. *Effect of growth cycle*

As the different isozyme pattern was observed under various physiological conditions, the changes in isozyme pattern were followed during the growth cycle of the bacteria. Overnight culture of strain C90, a *phoT* mutant,

was diluted ten-fold into fresh T broth, and then an aliquot of the culture was withdrawn at the various phases of the growth cycle. Although the data are not presented, the isozyme 1 was predominantly found during early logarithmic phase of growth. As the culture came into stationary phase of growth, the isozyme 3 predominated as compared with isozyme 1 or 2.

4. *Effects of amino acids and glucose*

From the results described in the previous sections, it was not clarified whether a variety of isozymes were synthesized separately depending on a variety of cultural conditions or if they were in somewhat precursor-product relationships, in other words, only one form of isozyme was synthesized and then it was modified into other forms.

Schlesinger and Andersen (1968) reported that the isozyme 1 was first synthesized and then converted into isozyme 2 or 3, under the condition in which the isozyme 3 was detected predominantly. Piggott et al. (1972) reported that, in the presence of exogeneous arginine, the isozyme 1 was preferentially synthesized.

In order to see whether a variety of different isozymes are separately synthesized or one isozyme is synthesized and then converted to other forms, and if conversion is the case, to see whether it requires energy supply or not. And to confirm whether or not externally added arginine, its analog canavanine or casamino acids inhibits conversion of isozymes, the following experiment was carried out.

A culture of strain K10 was starved phosphate (derepression of the enzyme) in the presence of arginine (200 μg/ml). The bacterial cells were harvested and transferred into the medium supplemented with excess phosphate to prevent further synthesis of enzyme (repression of the enzyme), and then incubated with or without added glucose, arginine, canavanine or casamino acids. After further overnight incubation, the enzyme was extracted and its isozyme pattern was

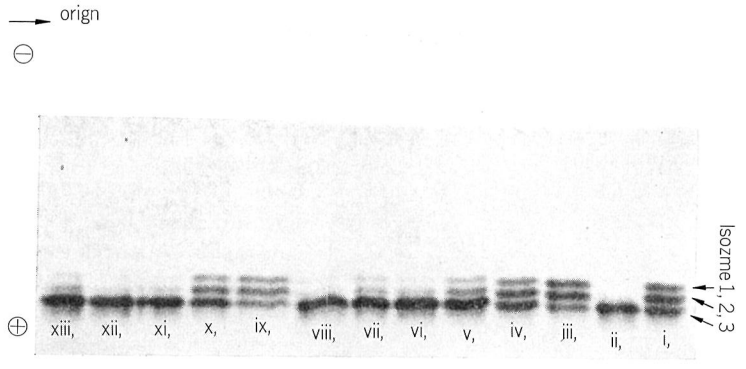

FIGURE 2. A 50 ml of culture of strain K10 in TGC medium supplemented with excess phosphate and arginine (200 μg/ml) was harvested by centrifugation during logarithmic phase of growth and resuspended in equal volume of TG medium supplemented with arginine (without added phosphate) and then incubated at 37C with shaking overnight (derepression of the enzyme). After overnight incubation, cells were collected by centrifugation and resuspended into 70 ml of T salts medium supplemented with excess phosphate (to repress further synthesis of the enzyme) and then divided into two portions, to one of which glucose was added but not to the other. Each portion was then divided 5 ml each into 6 tubes. To each tube was added arginine, canavanine or casamino acids, respectively. They were continued incubation with shaking overnight. After overnight incubation, the cells were harvested by centrifugation and resuspended into 2 ml Tris-Mg^{++} buffer, and the suspensions were sonicated. An aliquot of sonicates was applied on gel electrophoresis. i), enzyme sample syntehsized in the presence of arginine (before repression of the enzyme); ii) to xiii), after further incubation (repression of the enzyme after overnight derepression). ii), with glucose only; iii), with glucose and arginine (200 μg/ml); iv), same as iii) but arginine at 67 μg/ml; v), with glucose and canavanine (200 μg/ml); vi), same as v) but canavanine at 67 μg/ml; vii), with glucose and casamino acids (2 mg/ml); viii)=ii); ix)=iii); x)=iv); xi)=v); xii)=vi); xiii)=vii) but without added glucose. No increase of enzyme activity (or slight decline of it in the culture without glucose) was observed during repression of the enzyme.

analysed on electrophoresis. The results are shown in Fig. 2 and are summarized as follows: 1) Isozyme 3 is formed as the result of conversion of isozyme 1 or 2, but not synthesized separately. 2) Conversion from isozyme 1 to 3 (probably through isozyme 2) occurs after polymerization of polypeptides. 3) It does not require energy source. 4) It is prevented by exogeneous arginine but partially by canavanine or casamino acids.

5. *Isolation of mutant strain unable to produce isozymes*

We tried to isolate a mutant strain which does not convert (or converts very slowly) isozyme 1 to 2 or 3, under the conditions in which it is converted in wild type strain. As we had no way to select such a mutation directly, we mutagenized a culture of strain C90 heavily with NG, and allowed survived colonies to produce alkaline phosphatase in TG medium supplemented with limited phosphate and casamino acids (0.002% instead of 0.2%). Each bacterial culture was harvested and resuspended into Tris-Mg^{++} buffer, and the cells were disrupted with toluene. They were then heated at 90C for 15 to 20 min and then the supernatants were analysed on gel electrophoresis.

Of the 494 colonies tested, one strain (AN-234) synthesized isozyme 1 predominantly and a slight amount of isozyme 2, but a very slight amount of isozyme 3, if any, irrespective of a removal of casamino acids or phosphate from the medium (Fig. 3). Figure 3 shows that 1) in the presence of casamino acids, all three isozymes are found (synthesis of isozyme 1 seems to predominate depending on the duration of derepression of the enzyme), 2) isozymes 1 and 2 are converted into isozyme 3 irrespective of added phosphate, 3) the mutations in regulatory genes have no effect at all on the conversion of isozymes and 4) in mutant strain, isozymes 1 and 2 are produced but they are not converted into isozyme 3, under the conditions in which the conversion occurs in other strains.

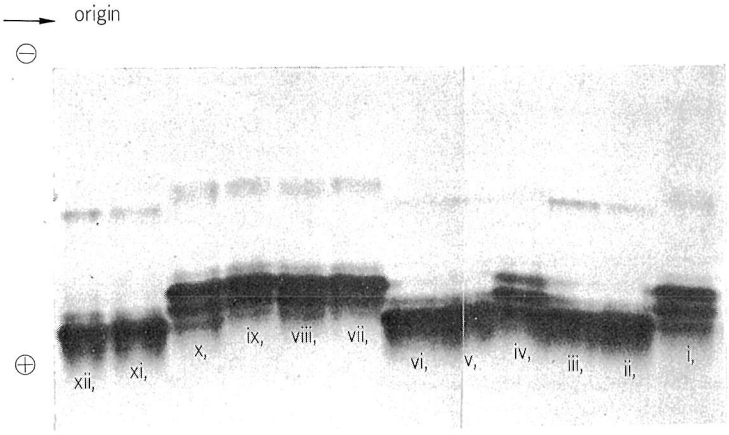

FIGURE 3. The cultures in TGC medium supplemented with limited (K10) or excess (AN234, C80 or C90) phosphate were harvested by centrifugation and resuspended into TG medium (without added casamino acids), and then incubated with or without supplemented excess phosphate overnight. Enzyme sample was prepared as described in legend to Fig. 2. i), K10 grown in TGC medium, before transfer to TG medium; ii), K10 after transfer to TG medium without phosphate; iii), same as ii) but with excess phosphate; iv), C80 grown in TGC medium, before transfer to TG medium; v), C80 after transfer to TG medium without phosphate; vi), same as v) but with excess phosphate; vii), AN234 grown in TGC medium, before transfer to TG medium; viii), AN234 after transfer to TG medium without phosphate; ix), same as viii) but with excess phosphate; x), C90 grown in TGC medium, before transfer to TG medium; xi), C90 after transfer to TG medium without phosphate; xii), same as xi) but with excess phosphate.

The fact that the mutation affecting isozyme formation did not occur in aklaline phosphatase structure gene (*phoA*) was proved by the following experiments. The bacteriophage P1 lysate was prepared on the strain AN234 and transduction experiment was carried out with LC607 as a recipient. No Pro^{+} selected transductants showed the mutational isozyme pattern like a donor strain (co-transduction frequency of *pro*C gene with

phoA gene is over 84%; Nakata et al., 1971). The results of genetic recombination experiment between AN234 and LC607 suggested that the mutation occurred somewhere between *trp* and *metE* genes. A recombinant strain of this genetic cross and showing mutational isozyme pattern (produces isozyme 1 and slight amount of 2 in either TGC or TG medium) but still having *proC*$^-$ genotype was infected with P1 phage prepared on strain U24R3Ua which produces alkaline phosphatase moving faster than the wild type enzyme on electrophoresis, and then Pro$^+$ transductants were selected. Most of Pro$^+$ selected transductants showed the isozyme pattern characteristic of recipient strain, but moving faster than the wild type enzyme, as donor strain, on electrophoresis. On the other hand, when a recombinant strain showing wild type isozyme pattern from the above mentioned genetic cross was transduced with the same P1 lysate, most of Pro$^+$ selected transductants produced all three isozymes in TGC medium and only isozyme 3 in TG medium, but all of them moved faster than the wild type enzyme on electrophoresis.

We, therefore, tentatively propose to designate the gene(s) controlling isozyme formation as *iap*, short for *i*sozyme of *a*lkaline *p*hosphatase. Precise genetic mapping of the mutation(s) and the mode of action of the *iap*$^+$ gene(s) are under investigation.

DISCUSSION

It is well known that alkaline phosphatase in *E. coli* is a dimer composed of homologous polypeptides, which is coded for by a single structure gene (Garen and Garen, 1963; Rothman and Byrne, 1963), nevertheless, highly purified enzyme is sometimes separated into mainly three (occasionally four or more) enzymatically active forms on starch gel or polyacrylamide gel electrophoresis (Signer et al., 1961; Levinthal et al., 1962; Malamy and Horecker, 1964; Lazdunski and Lazdunski, 1967). Of these isozymes, it is also reported that each of the isozyme 1 and 3 is a dimer composed of homologous polypeptides, but the isozyme 2 is composed of one monomer of isozyme 1 and one monomer of isozyme 3 (Lazdunski and Lazdunski, 1967; Schlesinger and Andersen, 1968).

Several mechanisms have been proposed to explain the formation of these multiforms of the enzyme. These are either ambiguous translation of a genetic message during polypeptide synthesis or modification of enzyme molecules after synthesis and dimerization of polypeptides.

In this paper, we confirmed that the isozyme 1 was first synthesized and then converted into isozyme 3 through isozyme 2, as has been reported previously (Schlesinger and Andersen, 1968). The fact that the enzyme synthesized in the absence of casamino acids is almost exclusively isozyme 3 suggests that the conversion occurs very rapidly under the condition. And it is likely that the isozyme 3 found predominantly in the enzyme producing bacteria, during stationary phase of growth, is an accumulation of final products resulting from conversion of other forms. We also confirmed the conversion was effectively prevented by exogeneously added arginine (Piggott et al., 1972; Schlesinger et al., 1975), but partially by canavanine and casamino acids. Chloramphenicol, trypsin inhibitor or diisopropyl fluorophosphate had no effect at all on the conversion of isozymes (unpublished data).

Kelley et al. (1973) reported that, in amino-terminal fragment obtained from cyanogen bromide cleavage of *E. coli* alkaline phosphatase, the amino-end arginine residue is present in isozyme 1, but not in isozyme 3. Thus, they inferred that the conversion from isozyme 1 to isozyme 3 occurs by protease after the alkaline phosphatase subunits have dimerized and attained catalytically-active conformation (Schlesinger et al., 1975).

As it is well recognized that the enzymatically active alkaline phosphatase locates in periplasmic region (Malamy and Horecker, 1961; Neu and Heppel, 1964; Heppel, 1971), and

the conversion of isozymes occurs after the enzyme have dimerized, it is very likely that the postulated protease also locates in the same region, and its synthesis is under a regulation completely different from alkaline phosphatase synthesis, because neither energy source nor *de novo* synthesis of the alkaline phosphatase is required for the conversion.

We isolated an *E. coli* mutant strain unable to produce, if any, isozyme 3. The mutation(s) was proved to occur not in alkaline phosphatase structure gene but somewhere in between *trp* and *metE* genes of *E. coli* genetic map. Therefore, we proposed to designate the mutation(s) as *iap*, short for *i*sozyme of *a*lkaline *p*hosphatase. This mutation(s) is thought to be either defective of a proteolytic enzyme, mentioned in the previous paragraphs, or defective in some unknown process of conversion. It is not concluded yet that the mutation occurred in one cistron, unless the detailed genetic mapping is completed. The possibility that the *iap* mutation may be a multiple mutations in distinct cistrons is not ruled out, because so many mutations were found from the same mutagenized culture of the bacteria (Table 2). The number of mutation in Table 2 is still underestimated, because, as described in the section of materials and methods, single colony isolation has been carried out before a possible mutation has completely segregated.

The above mentioned mechanism (conversion of isozyme 1) seems insufficient to explain the formation of two additional isozymes occasionally found ahead and behind of the main three isozymes on electrophoresis (see, Fig. 3). For instance, deamination of asparagine or glutamine in the enzyme molecule as suggested in isozymic variation of colicin E_3 (Glick et al., 1972) or attachment of carbohydrates to polypeptides as suggested by Reid and Wilson (1971), or even miss-translation during polypeptide synthesis as the results of mutational changes in 30s ribosomal proteins (Piggott et al., 1972) might occur in addition to the removal of amino acids from the enzyme molecules.

TABLE 2. *Mutations in alkaline phosphatase structure gene (phoA) obtained from a mutagenized culture of C90*

Total colonies tested	494
Fast moving[a]	2
Slow moving[a]	3
Negative[b]	3
Isozyme (*iap*)[c]	1

[a] Alkaline phosphatase synthesized migrates either faster or slower than the wild type enzyme on electrophoresis.

[b] Mutation unable to synthesize alkaline phosphatase which would be mapped either in *phoA* or *phoB* (or R^c) gene.

[c] Mutation occurred not in *phoA* gene, see text.

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