Alkaline phosphatase isozyme conversion by cell-free extract of *Escherichia coli*

Atsuo Nakata, Hideo Shinagawa and Hisaharu Shima

Department of Experimental Chemotherapy, The Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka, Japan 565

Received 1 July 1984; revised version received 8 August 1984

Isozyme type 1 of alkaline phosphatase in *Escherichia coli* K-12 was converted to types 2 and 3 after incubation of type 1 isozyme with the supernatant of a sonicated cell-free extract prepared from the cells carrying the cloned *iap*$^+$ gene on a multi-copy plasmid. By comparison, the lysate prepared from cells carrying the *iap*$^+$ gene only on the chromosome showed much less isozyme-converting activity. The reaction was promoted by Mg^{2+} at concentrations of 10 to 50 mM. Protease inhibitors, antipain and leupeptin which inhibit the isozyme conversion in vivo, also inhibited the isozyme conversion in vitro. These results suggest that cells carrying the multiple copy *iap*$^+$ plasmid overproduce a kind of proteolytic enzyme which removes the amino-terminal arginine residues from isozymes 1 and 2.

Escherichia coli *Alkaline phosphatase isozyme* *In vitro isozyme conversion* *Cloned* iap$^+$ *gene* *Proteolytic enzyme* *Protease inhibitor*

1. INTRODUCTION

Enzymatically active alkaline phosphatase (EC 3.1.3.1), which is coded by the *phoA* gene in *Escherichia coli* K-12, consists of two homologous polypeptides [1]. Nevertheless, when the purified enzyme sample was analyzed by electrophoresis on polyacrylamide gel in the absence of sodium dodecylsulfate (SDS) and stained for enzymatic activity, three major isozyme bands were occasionally observed [2–7]. An isozyme pattern with all three main bands was observed when the alkaline phosphatase-synthesizing cells were cultured in a medium supplemented with casamino acids or arginine [7–9]. The isozyme pattern was converted to one with only isozyme 3, the fastest moving band on the gel during electrophoresis, when the cells were further cultured in an excess phosphate medium without the supplements, which prevents further synthesis of alkaline phosphatase [8,11]. The difference between isozyme 1, the slowest moving band of the three, and isozyme 3, the fastest band, is the presence of amino-terminal arginine residues in isozyme 1 and its absence in isozyme 3 [10–13]. Isozyme 2, the middle band, is a heterologous dimer composed of one polypeptide of isozyme 1 and one polypeptide of isozyme 3 [5]. Thus the conversion from isozyme 1 to isozymes 2 and 3 might be brought about by the proteolytic removal of the amino-terminal arginine residues from isozymes 1 and 2. It was reported that the isozyme conversion in vivo is inhibited by arginine or some protease inhibitors [7,9].

We isolated *E. coli* mutants which produce mainly isozyme 1 under culture conditions which enabled the wild-type cells to produce only isozyme 3 [8,13]. The mutation, tentatively designated as *iap* (an abbreviation of isozyme of alkaline phosphatase) was mapped at 59 min on the standard *E. coli* genetic map [14]. The *iap*$^+$ gene on the F-prime factor was dominant over the *iap*$^-$ mutation on the chromosome [15]. We cloned an *E. coli* chromosomal DNA fragment, which complements the *iap*$^-$ mutation, on plasmid pBR322 and showed that the cloned DNA fragment contains the authentic *iap*$^+$ gene [16]. Isozyme conversion in

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

cells carrying the *iap*$^+$ plasmid was not inhibited by arginine at a concentration which inhibited the conversion in control cells without the plasmid. These results indicate that in cells carrying the multi-copy *iap*$^+$ plasmid, a kind of proteolytic enzyme, presumably encoded by the *iap* gene, is overproduced, which removes the amino-terminal arginine residues from isozymes 1 and 2.

As the first step to identify and purify the enzyme responsible for the isozyme conversion, we attempted to set up an in vitro isozyme conversion system by taking advantage of the Iap overproducing strain. We report the isozyme conversion by cell-free extracts and some preliminary studies on the physiological conditions for the conversion.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli K-12 strain FE15 was constructed by conjugation between strains E15 [17] and P678 [17], and PhoA$^-$, Lac$^-$, Thr$^-$, Leu$^-$, Thi$^-$ and Str^R recombinant was selected. It has a deletion mutation within the *phoA* gene [18]. Strain ANJ426 (F$^-$ *leu-6 his-1 cysC43 iap-1 strA104 metB*) has been described previously [13].

Plasmids pBR322 and pSN143 containing the *iap*$^+$ gene on pBR322 were described previously [16].

2.2. *Culture medium*

A TG medium, consisting of a minimal salt solution buffered with 0.12 M Tris–HCl at pH 7.2 and containing 0.2% glucose was supplemented with excess (10^{-3} M) phosphate (as KH_2PO_4) [19]. Amino acids, thiamine and antibiotics, when needed, were added to the medium as described previously [20,21]. Casamino acids were added at a concentration of 0.02%.

2.3. *Chemicals*

The protease inhibitors antipain and leupeptin, were donated by H. Umezawa, Institute of Microbial Chemistry, Tokyo. Ovomucoid (egg white) trypsin inhibitor was purchased from Sigma.

2.4. *Preparation of alkaline phosphatase isozyme 1*

The isozyme 1 of alkaline phosphatase was isolated from cells of strain ANJ426 (*iap*$^-$) and purified by diethylaminoethyl-cellulose (DE52, Whatman) column chromatography as described previously [13]. It was dialysed against a 50 mM Tris–HCl (pH 8.0) buffer containing 0.02% NaN_3 and concentrated by using a collodion bag to a final concentration of 560 enzyme units/ml. The assay method for alkaline phosphatase activity was described previously [22]. One enzyme unit is expressed as 1 μmol of *p*-nitrophenol liberated per min at 37°C.

2.5. *Preparation of cell-free extracts*

Crude cell-free lysates for isozyme conversion were prepared from cells of strain FE15 carrying either pSN143 or pBR322; since this strain has a deletion in the *phoA* gene, the corresponding cell extracts prepared do not contain alkaline phosphatase. The cells grown in TG medium supplemented with excess phosphate, required amino acids, antibiotics, and casamino acids were transferred into TG medium without phosphate during the exponential phase of growth. After incubation at 37°C overnight with shaking, the cells were harvested and washed once with 50 mM Tris–HCl (pH 8.0) buffer containing 0.02% azide. The cells were resuspended in the same buffer containing 0.02% azide and adjusted to a density of 150 A_{540nm} units/ml. The cell suspension was sonicated in an ice-water bath, and cellular debris and undisrupted cells were removed by centrifugation at 8000 rpm in a Sorvall SS34 rotor for 30 min. The supernatant was stored at −20°C until use.

2.6. *Reaction conditions for isozyme conversion and examination of isozyme pattern*

The reaction mixture (200 μl) for isozyme conversion in vitro contained 50 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$ and 0.02% NaN_3 unless otherwise stated, as well as 10 μl of isozyme 1 (5.6 enzyme units) and 150 μl of cell lysate. The final concentration of $MgCl_2$ and azide were 20 mM and 0.02%, respectively. The reaction mixture was incubated at 37°C and the reaction terminated by heating at 80°C in a water-bath for 10 min. The denatured proteins were removed by centrifugation and the supernatant was applied to a gel for electrophoresis. For the detection of isozyme conversion, polyacrylamide gel (7.5%) electrophoresis

was carried out and the gel was stained for alkaline phosphatase activity as described previously [16].

3. RESULTS AND DISCUSSION

Previously [16] we constructed a hybrid plasmid, pSN143, by cloning the *E. coli* chromosomal DNA fragment containing the *iap*$^+$ gene on a multiple copy-number plasmid vector pBR322. As a high gene-dose effect of the *iap*$^+$ gene responsible for isozyme formation was expected, a crude cell lysate for in vitro isozyme conversion was prepared from a culture of strain FE15 carrying pSN143. An aliquot of the crude cell lysate was mixed with purified alkaline phosphatase which contained mainly isozyme 1, and the mixture was incubated at 37°C. As shown in fig.1, isozyme 1 was converted to isozymes 2 and 3 during incubation. The conversion did not occur in the absence of cell lysate (see fig.4, lane 1) or when the mixture was heated at 80°C for 10 min prior to incubation (fig.1, lane 6).

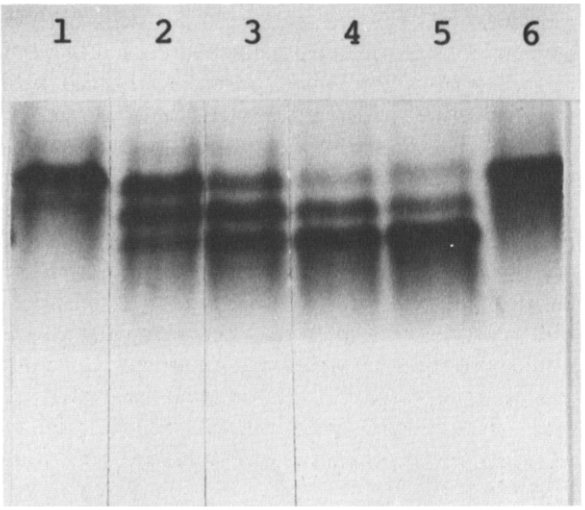

Fig.1. Conversion of alkaline phosphatase isozyme by cell-free extract. The reaction mixture (1.5 ml) contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.02% azide, 0.1 ml alkaline phosphatase isozyme 1 (56 enzyme units) and 1.25 ml of crude cell lysate prepared from FE15 carrying plasmid pSN143. The reaction mixture was incubated at 37°C. An aliquot of 0.125 ml reaction mixture was withdrawn at the times indicated below, and the reaction stopped by heating at 80°C for 10 min. The sample was centrifuged at 8000 × *g* for 5 min, and to 80 μl of the supernatant were added 20 μl of glycerol containing phenol red (1%). A 20-μl portion of the mixture was used for electrophoresis. Samples were withdrawn at 0 min (lane 1), 30 min (lane 2), 60 min (lane 3), 90 min (lane 4) and 120 min (lane 5). The sample of lane 6 was heated at 80°C for 10 min before incubation for 120 min.

To optimize the reaction conditions, we examined the effects of some metal ions on isozyme conversion. The reaction was stimulated by Mg^{2+} (as $MgCl_2$) at concentrations of 10 to 50 mM (fig.2, lanes 1–5), but less so by Ca^{2+} (as $CaCl_2$) in the same concentration range (fig.2, lanes 8–12). KCl had no additive stimulatory effect to Mg^{2+} on the reaction (fig.2, lanes 13–17). Mn^{2+} (as $MnCl_2$) showed similar levels of stimulatory effect to Mg^{2+} (not shown). Hereafter, $MgCl_2$ was added at a concentration of 20 mM to the reaction mixture.

To examine the optimal pH range for isozyme conversion, the lysates were dialyzed against 50 mM Tris–HCl at pH 8.5, 8.2, 8.0, 7.8, 7.5 or 7.2 (each containing 0.02% azide) at 4°C overnight, and the reaction carried out at the corresponding pH levels. The isozyme patterns obtained by using these dialysates are shown in fig.3. The most efficient isozyme conversion was observed when the lysate was dialyzed against the buffer of pH 7.8, in which most of isozyme 1 was converted to isozymes 2 and 3 by incubation of the mixture overnight (fig.3, lane 5). Efficient conversion occurred with the lysates dialyzed against buffers at pH levels ranging from 8.0 to 7.2 (lanes 4–7), but less efficient conversion was observed with the lysates dialyzed against buffers of pH 8.5 or 8.2 (lanes 2 and 3).

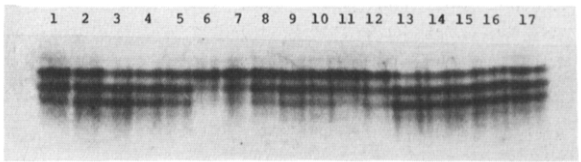

Fig.2. Effect of $MgCl_2$, $CaCl_2$ and KCl on isozyme conversion in vitro. The samples in lane 6 contained no lysate and lane 7 contained lysate without the addition of metal ion. The reaction mixtures in lanes 1–5 contained 5, 10, 25, 50 and 100 mM $MgCl_2$, respectively; those in lanes 8–12, 5, 10, 25, 50 and 100 mM $CaCl_2$, respectively; and those in lanes 13–17, 20 mM $MgCl_2$ together with 5, 10, 25, 50 and 100 mM KCl, respectively. Reaction mixtures were incubated overnight.

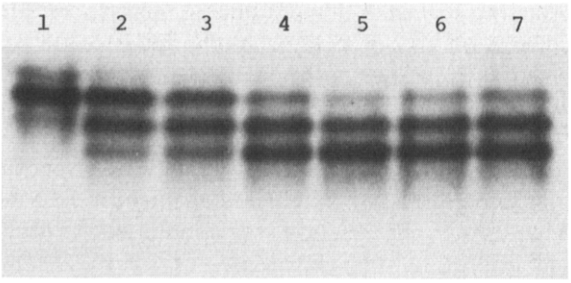

Fig.3. Effect of pH on isozyme conversion in vitro. The cell lysate was divided into 6 portions. Each portion was dialyzed against 50 mM Tris–HCl at pH levels of 8.5, 8.2, 8.0, 7.8, 7.5 and 7.2, containing 0.02% azide. Isozyme conversion was carried out at the corresponding pH levels, and the reaction stopped after 4 h incubation. The sample in lane 1 contained a buffer of pH 7.5 instead of cell lysate. Those in lanes 2–7 were of pH 8.5, 8.2, 8.0, 7.8, 7.5 and 7.2, respectively.

The finding that more extensive conversion occurred during incubation of the mixture overnight than incubation for 3 or 4 h (see figs 5 and 6) indicates that the conversion process was slow and the lysates retained the converting activity even after dialysis against the buffer and prolonged incubation. The isozyme converting activity of the lysate was retained for at least 3 months at −20°C (see fig.6, lane 10).

In a previous study [9], it was observed that isozyme conversion within the cell was inhibited by arginine and some protease inhibitors such as antipain and leupeptin. The effect of these inhibitors on isozyme conversion in vitro was examined (fig.4). Almost no isozyme conversion was observed in the presence of 0.11 mM leupeptin (fig.4, lane 8). Antipain also showed an inhibitory effect on the conversion (lanes 3–5), but less than leupeptin. The effect of these agents on the conversion was detectable at lower doses with the in vitro system than with the in vivo system [9]. Inhibition by arginine was not very apparent when the mixture was incubated for 6 h (lanes 9–11), but was more apparent when the reaction was terminated at 3 h (not shown). The inhibitory effect of arginine on isozyme conversion may be due to end-product inhibition [11], or to competitive inhibition, since the conversion is also inhibited by some oligopeptides containing L-arginine [9]. Since the inhibitory effect of arginine was detectable at lower doses with the in vivo system [9] than with the in vitro system, it is possible that synthesis of the enzyme responsible for isozyme conversion is repressed by arginine added to the medium. The trypsin inhibitor, which showed no effect on isozyme conversion in vivo, also did not inhibit the conversion in vitro (fig.4, lanes 12–14).

The cell-free isozyme conversion experiments described above were all carried out with extracts of the cells carrying a multi-copy *iap*$^+$ plasmid, pSN143. Since [16] suggests that the conversion in vivo is more efficient in cells carrying the multi-copy *iap*$^+$ gene than in cells carrying the single

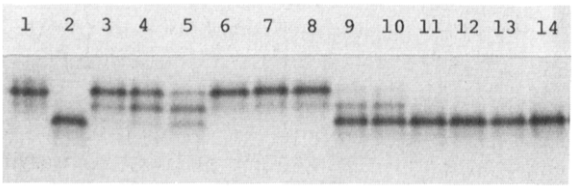

Fig.4. Effect of protease inhibitors and arginine on isozyme conversion. The reaction mixtures were incubated for 6 h. Lane 1: without cell lysate; 2: without inhibitor; lanes 3–5: 1, 0.33 and 0.11 mM antipain, respectively; lanes 6–8: 1, 0.33 and 0.11 mM leupeptin, respectively; lanes 9–11: 3, 1 and 0.33 mM arginine, respectively; lanes 12–14: 300, 100 and 33 μg/ml of ovomucoid (egg white) trypsin inhibitor, respectively.

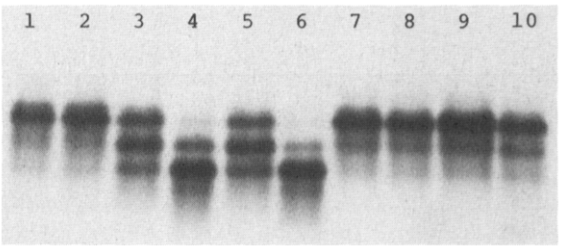

Fig.5. Effect of *iap*$^+$ gene dosage and culture conditions on isozyme conversion. The reaction mixture (400 μl) contained 50 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 0.02% azide, 20 μl isozyme 1 (11.2 enzyme units), and 340 μl cell lysate. Lysates were prepared from cells of strain FE15 carrying pBR322 grown in excess phosphate medium (lanes 7 and 8) or in phosphate-deprived medium (lanes 9 and 10), and from cells of strain FE15 carrying pSN143 grown in excess phosphate medium (lanes 3 and 4) or in phosphate-deprived medium (lanes 5 and 6). In lanes 1 and 2, the buffer was added instead of cell lysate. Incubation time was 3 h (lanes 1,3,5,7 and 9) and overnight (lanes 2,4,6,8 and 10).

copy gene, we examined the gene-dosage effect on the conversion activity in vitro. As shown in fig.5, very efficient isozyme conversion occurred with extracts prepared from cells of FE15/pSN143 (lanes 3–6) while no apparent conversion was detected in the reaction mixture with extracts from cells of FE15/pBR322 (lane 7–10). Thus the demonstration of isozyme conversion in vitro was accomplished only with the extract from the cells carrying the multi-copy plasmid containing the *iap*$^+$ gene.

Since the substrate of the conversion enzyme is alkaline phosphatase and the synthesis of the enzyme is induced by phosphate starvation, the conversion enzyme may also be inducible by phosphate limitation. We compared the conversion activity of the extracts prepared from the cells of FE15/pBR322 and those of FE15/pSN143 grown in excess phosphate and phosphate-deprived media (fig.5). Marginally more efficient conversion was obtained by the extracts of the cells grown under phosphate-deprived conditions (lanes 5 and 6) than by those of the cells grown in excess phosphate medium (lanes 3 and 4). However, the difference in efficiency was so small that we could not conclude that the synthesis of the conversion enzyme was induced by phosphate starvation.

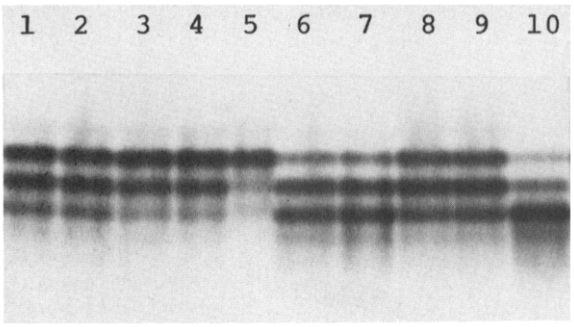

Fig.6. Effect of lysate concentration, casamino acid concentration and incubation time on isozyme converting activity. Lysates were prepared from cells grown in medium with 0.02% (lanes 1,2,6 and 7) or with 0.2% (lanes 3,4,8 and 9) casamino acids. The samples in lanes 1,3,6 and 8 contained 150 μl lysate and those in lanes 2,4,7 and 9, 100 μl lysate per 200 μl reaction mixture. Reaction mixtures were incubated for 4 h (lanes 1–4) and overnight (lanes 6–10). The sample in lane 5 contained no lysate. In the sample in lane 10, the reaction was performed with a cell lysate prepared from FE15/pSN143 cells grown on a phosphate-deprived medium supplemented with 0.02% casamino acids, and stored at −20°C for 3 months.

Since the lysate with more efficient converting activity was prepared from the cells cultured in a medium supplemented with a low concentration of casamino acids (0.02% instead of 0.2%) as shown in fig.6, the *iap*$^+$ may be expressed when the amino acids in the medium have been exhausted by the cells.

This study provides a system to assay the putative isozyme conversion enzyme in vitro, and opens the way to the purification of the enzyme responsible for the conversion. Although all the evidence obtained so far is consistent with the hypothesis that *iap*$^+$ encodes the proteolytic enzyme which cleaves the amino-terminal arginine of alkaline phosphatase, we cannot exclude other possibilities, e.g., that it encodes a positive regulator of the structural gene of the conversion enzyme, or an activator for the enzyme. Identification of the *iap*$^+$ gene product on the one hand and the purification of the conversion enzyme on the other will ultimately answer this question.

REFERENCES

[1] Rothman, F. and Byrne, R. (1963) J. Mol. Biol. 6, 330–340.
[2] Garen, A. and Levinthal, C. (1960) Biochim. Biophys. Acta 38, 470–483.
[3] Signer, E.R., Torriani, A. and Levinthal, C. (1961) Cold Spring Harbor Symp. Quant. Biol. 26, 31–34.
[4] Levinthal, C., Signer, E. and Fetherolf, K. (1962) Proc. Natl. Acad. Sci. USA 48, 1230–1237.
[5] Lazdunski, C. and Lazdunski, M. (1967) Biochim. Biophys. Acta 147, 280–288.
[6] Schlesinger, M.J. and Andersen, L. (1968) Ann. NY Acad. Sci. 151, 159–170.
[7] Piggot, P.J., Sklar, M.D. and Gorini, L. (1972) J. Bacteriol. 110, 291–299.
[8] Nakata, A., Amemura, M., Yamaguchi, M. and Izutani, K. (1977) Biken J. 20, 47–55.
[9] Nakata, A., Shinagawa, H. and Kawamata, J. (1979) FEBS Lett. 105, 147–150.
[10] Kelley, P.M., Neumann, P.A., Shriefer, K., Cancedda, F., Schlesinger, M.J. and Bradshaw, R.A. (1973) Biochemistry 12, 3499–3503.
[11] Schlesinger, M.J., Bloch, W. and Kelley, P.M. (1975) in: Isozymes I. Molecular Structure (Markert, C.L. ed.) pp.333–342, Academic Press, New York.

[12] Bradshaw, R.A., Cancedda, F., Ericsson, L.H., Neumann, P.A., Piccoli, S.P., Schlesinger, M.J., Shriefer, K. and Walsh, K.A. (1981) Proc. Natl. Acad. Sci. USA 78, 3473–3477.
[13] Nakata, A., Yamaguchi, M., Izutani, K. and Amemura, M. (1978) J. Bacteriol. 134, 287–294.
[14] Bachmann, B.J. (1983) Microbiol. Rev. 47, 180–230.
[15] Nakata, A. and Kawamata, J. (1980) Biken J. 23, 49–51.
[16] Nakata, A., Shinagawa, H. and Amemura, M. (1982) Gene 19, 313–319.
[17] Bachmann, B.J. (1972) Bacteriol. Rev. 36, 525–557.
[18] Sarthy, A., Michaelis, S. and Beckwith, J. (1981) J. Bacteriol. 145, 288–292.
[19] Nakata, A., Peterson, G.R., Brooks, E.L. and Rothman, F.G. (1971) J. Bacteriol. 107, 683–689.
[20] Davis, R.W., Botstein, D. and Roth, J.R. (1980) in: Advanced Bacterial Genetics. A Manual for Genetic Engineering, p.206, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning. A Laboratory Manual, p.444, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[22] Amemura, M., Shinagawa, H., Makino, K., Otsuji, N. and Nakata, A. (1982) J. Bacteriol. 152, 692–701.