SYNTHESIS OF INACTIVE β-GALACTOSIDASE DURING AMINO ACID STARVATION IN *ESCHERICHIA COLI* K-12

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1. Introduction

Premature termination of nascent proteins has been found to be negligible after a few minutes of the onset of amino acid starvation in *Escherichia coli* K-12 [1]. Proper completion of proteins is also evident from the detection of activity of enzymes like tryptophan synthetase [2], alkaline phosphatase [3] and ornithine transcarbamylase [4], synthesized during amino acid starvation. However, anomaly exists, in so far as β-galactosidase activity fails to be properly induced during such starvation in *E. coli* [5]. We report here on the detection of the synthesis of inactive β-galactosidase during amino acid starvation and on the evidence suggesting complete but incorrect translation of the gene.

2. Materials and methods

2.1. *Organism and growth conditions*

Two strains of *Escherichia coli* K-12 (W3110) RC^+, one auxotrophic for leucine and proline and the other for leucine, proline and histidine have been used. Cells were grown at 37°C in a medium adopted from Goldstein et al. [6], but glucose was replaced by glycerol. Cells were normally harvested at a density similar to that in mid-log phase (A_{420}, 0.2). Details about amino acid starvation have been described earlier [1].

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2.2. *Induction and assay of β-galactosidase activity*

Cells were induced with IPTG (0.5 mM) in the presence of cyclic AMP (0.5 mM). β-Galactosidase activity (in 0.5 ml samples) was assayed according to Pardee et al. [7].

2.3. *Preparation of antiserum for β-galactosidase*

Authentic β-galactosidase (EC 3.2.1.23, Sigma, 200 μg/ml) in sodium phosphate buffer (0.01 M, pH 7.0) was mixed with equal volume of Freund's adjuvant and injected (1 ml) intramuscularly into a rabbit at weekly intervals. The fourth and last injection was given intravenously without the adjuvant. Bleeding was initiated 15 days later. The serum was obtained and stored at 4°C with 50% glycerol.

2.4. *Preparation of cell extract*

Cells were harvested, washed thrice with sodium phosphate buffer (0.01 M, pH 7.0) and resuspended in the same buffer. Lysis was done by ultrasonic oscillation in the MSE-Mullard Ultrasonic Disintegrator. The lysate was centrifuged at 20 000 × *g* for 10 min and the supernatant solution was used for reaction with antiserum.

2.5. *Determination of cross-reacting material in the cell extract*

Material in the cell extract capable of cross reacting with antiserum of β-galactosidase was determined according to Berg and Zabin [9].

2.6. *Electrophoresis of the material which cross reacts with antiserum of β-galactosidase*

Cells were concentrated four-fold by harvesting and

resuspending in a smaller volume of medium lacking leucine and proline. Incubated for 30 min at 37°C and divided into 4 portions of 5 ml each.

(i) [^{14}C]Leucine (10 μCi, 66 Ci/mol) and [^{14}C]arginine (10 μCi, 120 Ci/mol) added along with unlabelled proline. Some leucine (20 μg) and arginine (18 μg) were also added, bringing the specific radioactivity to half and allowing for longer incorporation. This would serve as non starved, uninduced control.

(ii) Same as (i) but IPTG (5×10^{-3} M) and cyclic AMP (5×10^{-3} M) were also added. Serves as non starved, induced control.

(iii) Same as (i) but no unlabelled proline, leucine or arginine added. Serves as proline-starved, uninduced control.

(iv) Same as (ii) but no unlabelled proline, leucine or arginine added. This is the proline-starved, induced set.

All the samples were incubated at 37°C under aeration for 20 min, the reaction was stopped by the addition of unlabelled leucine and arginine (1 mg/ml each) and chloramphenicol (200 μg/ml) and chilling in ice. The cells were harvested, washed with chilled medium, lacking amino acids, resuspended in sodium phosphate buffer (0.6 ml, 0.01 M, pH 7.0) and cell extract prepared. Authentic β-galactosidase (20 μg) was mixed with the cell extract (0.5 ml) and treated with the antiserum (0.2 ml). The immuno-precipitate was washed with normal saline, suspended in the same buffer (0.2 ml) containing 1% SDS and incubated for 10 min at 90°C. The total contents along with a small wash was electrophoresed according to Weber and Osborn [10] except that acrylamide concentration in gels was 10% and the relative proportion of bisacrylamide was half. Electrophoresis was done for 4 h at 6 mA/gel. Protein bands were detected by staining with Coomassie Blue. Radioactivity in the cut bands was determined in Bray's scintillation mixture [11] after digestion in capped vials with H_2O_2 for 10 min an autoclave at 15 p.s.i. Efficiency was 30%.

2.7. *EDTA treatment of cells*

Cells were treated with EDTA (1 mM) to permeabilize them towards actinomycin D [8].

3. Results

3.1. *Effect of amino acid starvation on the synthesis of β-galactosidase and proteins in general*

No activity of β-galactosidase could be induced in cells starving of leucine or histidine. The situation was unaltered even if the induction was prolonged for 75 min. On the other hand, general protein synthesis, as measured by incorporation of labelled proline, continued at about 10% level under similar conditions.

3.2. *Synthesis of inactive β-galactosidase protein during amino acid starvation*

If inactive β-galactosidase is formed and happens to be CRM$^+$, we should be able to detect it immunologically. A given amount of anti-β-galactosidase serum was mixed with an arbitrarily fixed amount of cell extract and then titrated with authentic and active β-galactosidase. After incubation and centrifugation of the immuno-precipitate, enzyme activity was assayed in the supernatant solution. The shift in the equivalence point towards lesser amount of the enzyme, on replacement of uninduced cell extract by the induced one, indicates the presence of CRM$^+$ in the induced starved cells (fig.1). The extent of this shift has been found to be more or less reproducible.

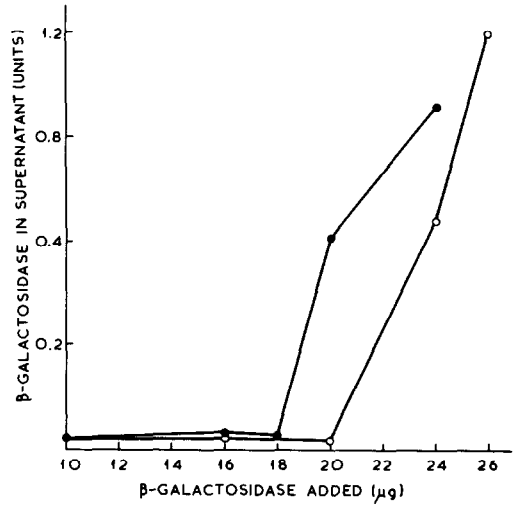

Fig.1. Formation of inactive, but CRM$^+$, β-galactosidase in starved cells. Cell extracts (0.1 ml) were mixed with graded doses of authentic β-galactosidase and incubated with a mixed amount of anti-β-galactosidase serum. The immuno-precipitate was centrifuged down and β-galactosidase activity in the supernatant solution was determined. (•)Leucine-starved cells induced for β-galactosidase; (○) uninduced leucine-starved cells.

Table 1
Incorporation of labelled amino acids into β-galactosidase protein

Culture		Radioactivity incorporated into total protein (dpm)	Radioactivity in β-galactosidase band (dpm)
(i)	Nonstarved uninduced	4.36×10^6	1.35×10^4
(ii)	Nonstarved induced	4.46×10^6	1.20×10^5
(iii)	Starved uninduced	1.27×10^6	1.30×10^3
(iv)	Starved induced	1.30×10^6	3.36×10^4

Specific radioactivities of labelled amino acids used in nonstarved culture were half of that used in starved cultures.

The immuno-precipitate was fractionated by SDS–acrylamide gel electrophoresis

3.3. *Electrophoresis of the CRM*$^+$

Immuno cross-reaction might as well be exhibited by prematurely terminated β-galactosidase protein. Hence, we have tested for the existence in the immuno-precipitate of material which co-electrophoreses with β-galactosidase. The data in table 1 indeed testify to the formation of such CRM$^+$ during amino acid starvation. We conclude, that full length, but enzymatically inactive, β-galactosidase proteins are synthesized on induction during amino acid starvation.

3.4. *Formation of inactive β-galactosidase is not due to incorrect transcription*

We speculate that the lack of enzymatic activity of β-galactosidase protein is because of mistakes, either during transcription or translation. If replenishment of the limiting amino acid to starved cells after inhibition of elongation of β-galactosidase messenger RNA by the addition of actinomycin D can elicit β-galactosidease activity, it can be inferred that β-galactosidase messenger RNA is transcribed correctly during amino acid starvation. A somewhat similar experiment has been reported [12]. We have rechecked this. Cells were first treated with EDTA and then starved of either leucine or histidine, since starved cells when treated with EDTA cannot be induced. Later, the cells were induced, samples were withdrawn periodically and incubated for 20 min with actinomycin D (20 μg/ml) plus the limiting amino acid. The cells were found to be sensitive to actinomycin D till at least 15 min after termination of EDTA treatment. β-Galactosidase activity was assayed after tolunization. It is evident that β-galactosidase messenger RNA is transcribed correctly during amino acid starvation (fig.2). If actinomycin D

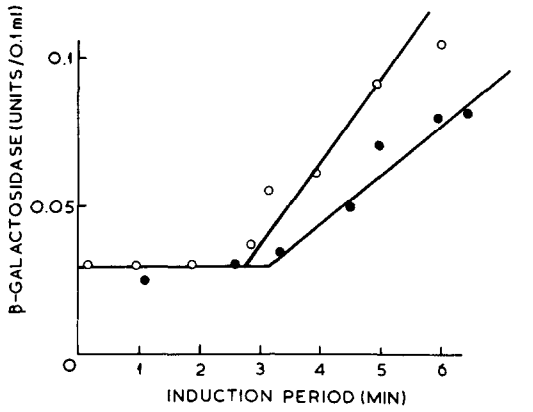

Fig.2. Correct transcription of β-galactosidase gene during amino acid starvation. EDTA treated cells were diluted and suspended in medium lacking leucine or histidine. Little later (4 min for leucine-starved and 6 min for histidine-starved cells) the cells were induced. Samples (0.1 ml) were withdrawn periodically, treated with actinomycin D (20 μg/ml) and the limiting amino acid was replenished after 1 min. Incubation was continued for an additional 20 min and β-galactosidase activity was determined. (○) Histidine-starved cells; (●) leucine-starved cells.

was added before the completion of transcription (about 3 min induction), no enzyme activity was detected even on prolonged incubation and restoration of the limiting amino acid. This has reassured us that inhibition by actinomycin D was complete.

We infer that mistakes if any, in the expression of the gene for β-galactosidase during amino acid starvation was at the level of translation.

4. Discussion

Amino acid starvation prolongs the waiting period of the ribosomes at the codons in messenger RNA. However, as long as the average step time (average interval between formation of two consecutive peptide linkages in the same protein) is within certain limits (upto about four times the normal), premature termination of nascent proteins does not occur and complete proteins are made [1]. Nevertheless, there might be some probability of the incorporation of a wrong amino acid once in a while, when the ribosome is unable to get the right one, in spite of considerable waiting. Obviously, the number of such wrong amino acids would be more per protein molecule, the bigger it is in size. The β-galactosidase monomer is an unusually long protein and hence the probability of mistakes occurring, at any of the large number of occasions when a ribosome confronts the codons of the limiting amino acid, would be comparatively higher. We speculate that this is the reason why, unlike some smaller proteins, β-galactosidase formed during amino acid starvation is inactive, in spite of its being complete.

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