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ENZYME SECRETION IN E. COLI K12 : STUDIES ON ALKALINE PHOSPHATASE SYNTHESIS USING AN UNSATURATED FATTY-ACID AUXOTROPH.

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SUMMARY: The role of unsaturated fatty-acid starvation, and of the substitution of trans for cis fatty acids in the membrane phospholipid, on the secretion of alkaline phosphatase, has been investigated. A system in which alkaline phosphatase synthesis was initiated by a temperature shift has been used to obviate possible complications resulting from phosphate depletion. In contrast to earlier reports, we find (a) there is very little effect of unsaturated fatty-acid starvation on the synthesis of alkaline phosphatase; (b) the synthesis of both β-galactosidase and alkaline phosphatase synthesis was severely reduced below 27-30°C in cells grown on trans Δ^9 16:1 fatty-acid, compared to cells grown on the cis Δ^9 16:1 analogue. Thus no preferential effect on alkaline phosphatase synthesis was observed.

The synthesis of membrane proteins, and particularly of the inducible lactose permease, have been studied in recent years using unsaturated fatty-acid auxotrophs (1,2,3). Such studies have been generally of two types: Experiments involving starvation for fatty-acid, and experiments involving the substitution of trans for cis fatty-acids. Experiments of the former type at first indicated that starvation for oleate resulted in cessation of incorporation of the lactose permease into the membrane (4), though later work refuted this conclusion (5). Studies on fatty-acid substitution show that after the onset of a phase transition in trans fatty-acid containing membranes, the incorporation (1,6,7) and function (see 1,8,9,10,11) of the lactose permease is impaired. The phase transition in cis fatty-acid containing membranes is usually too low to be as useful for correlating with physiological parameters.

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Corresponding experiments with a protein, alkaline phosphatase, which is secreted through the membrane, have been recently carried out (12,13). Alkaline phosphatase is a dimeric protein, but is secreted in the form of monomers; little or no enzyme is detectable inside the cell (14,15). The enzyme is repressed by inorganic phosphate and is derepressed by phosphate starvation (16). In order to study the role of the membrane lipid on secretion of alkaline phosphatase, new enzyme synthesis was initiated by resuspending cultures in medium lacking inorganic phosphate (12,13). It was found (12) that oleate starvation resulted in preferential cessation of alkakine phosphatase synthesis compared with a control, cytoplasmically located enzyme, β-galactosidase. It was also reported that cells grown on elaidic acid (trans 18:1), in contrast to oleic acid (cis 18:1) grown cells, were relatively deficient in their ability to synthesise alkaline phosphatase below the expected phase transition temperature for membrane lipid containing this trans fatty-acid, synthesis of β-galactosidase being much less affected. This result indicated that membrane fluidity was more important in the secretion process itself than in protein synthesis generally.

We have carried out similar studies on alkaline phosphatase synthesis, but using a temperature - sensitive system (17) to initiate synthesis of new enzyme.

EXPERIMENTAL

Strains and culture conditions. Strain KN238-2/19 has been described previously (17). It contains a phoR mutation which results in constitutive synthesis of alkaline phosphatase (i.e. in the presence of excess inorganic phosphate), an amber mutation in the structural gene (phoA) for alkaline phosphatase, and a temperature sensitive amber suppressor mutation (supD126). This strain makes alkaline phosphatase at a very low rate at 40°C, but at a high rate at 30°C (17, 18). The absolute amount of enzyme, however, made by amber suppression, is only a few percent of the amount made by phosphate depletion of a $phoA^+$ strain. This is presumably due to inefficient suppression. Two derivatives of strain KN238-2/19 were made as detailed in Table 1. One strain, KN238-2/19-10, through introduction of a fabB mutation is unable to synthesise unsaturated fatty-acids. The other resulting strain, designated KN238-146-14-10P, is unable to synthesise unsaturated fatty-acids(fabB), unable to degrade fatty-acids(fadE), and able to utilise trans fatty-acids efficiently. It might be emphasised that the fabB and fadE mutations are the same alleles as those used in previous studies involving fatty-acid replacement (8,9,19,20,21,22).

Table 1. Derivation of strains of Escherichia coli.

Strain	Genotype	Source/Reference
KN238-2/19	trpE tyr ilv supD126 tonA tsx, rpsL phoA (amS26) phoR17	(17)
KN238-2/19-10	trpE tyr ilv supD126 tonA tsx rpsL phoA (amS26) phoR17 nalA fabB	From KN238-2/19[a]
KN238-2/19-2	trpE tyr ilv supD126 tonA tsx rpsL phoA (amS26) phoR17 lac	From KN238-2/19[b]
KN238-146	trpE tyr supD126 tonA tsx rpsL phoA (amS26) phoR17 fadE	From KN238-2/19-2[c]
KN238-146-14	trpE tyr supD126 tonA tsx rpsL phoA (amS26) phoR17 fadE fabB nalA	From KN238-146[a]
KN238-146-14-10	trpE tyr supD126 tonA tsx rpsL phoA (amS26) phoR17 fadE fabB nalA	From KN238-146-14[d]
KN238-146-14-10P	supD126 tonA tsx rpsL phoA (amS26) phoR17 fadE fabB nalA	From KN238-146-14-10[e]
L35-2	HfrC fabB nalA	D.F. Silbert
YACI	HfrC fadE metB tsx rpsL λ^-	D.F. Silbert (25,26)

[a] By conjugation with L35-2 and selection for $NalA^+$. Exconjugants were scored for oleate requirement.

[b] Derived, using lactose-tetrazolium after mutagenesis with ethyl methane sulfonate.

[c] By conjugation with YACI and selection for Lac^+. Exconjugants were scored for inability to utilise oleate (1%) as a sole carbon source.

[d] A spontaneous mutant clone showing good growth on a trans fatty-acid (elaidic acid) was selected,

[e] A strain without amino-acid requirements derived from appropriate crosses. (This was done in case the restricted protein synthesis (see text) was due to difficulties in the uptake of amino-acids).

Genetic symbols are as defined in ref. 27.

Cultures were grown in a HEPES buffered medium (17) containing glycerol (0.2%) as carbon source and 0.1% Cas amino-acids.

Fatty-acids (obtained from Nu-Chek Prep., Minnesota) were made up in 80% ethanol, neutralised with KOH, and used at a final concentration of 100 µg/ml in the growth medium, which also contained 0.1% Brij 35.

Fatty-acid starvation. Cells were grown with oleate at 40°C to an $O.D._{450}$ of 0.4 ($\equiv 10^8$ cells/ml) and then spun, washed, and resuspended with or without oleate at an $O.D._{450}$ of about 0.1. Incubation was then continued at 30°C. Growth stopped abruptly after about 1½ generations. Samples (25ml) for measurement of alkaline phosphatase were taken and added at room temperature to tetracycline (final concentration, 50 µg/ml). They were then spun, washed, resuspended in buffer (1.0 TRIS, pH 8.0), disrupted by sonication and assayed as previously reported (17). Samples (1ml) were also added to 20µC of ^{14}C-acetate and labelled for 15 min. at 30°C to determine the instantaneous rate of endogenous fatty-acid synthesis. Incorporation into unsaturated and saturated fatty-acids was determined as described by Silbert (23). The rate of synthesis of unsaturated fatty-acid at intervals throughout these experiments was < 10% of that in strain KN238-2/19 (*fab* B^+). (The rate of synthesis of saturated fatty-acid was normal).

Fatty-acid replacement. Overnight cultures supplemented with oleate were spun, washed and resuspended in media containing palmitoleate (cisΔ^9 16:1) or palmitelaidate (*trans* Δ^9 16:1) and grown at 40°C for at least three generations. One generation is sufficient for almost complete fatty-acid replacement (20). β-galactosidase was induced with isopropylthiogalactoside (1mM) for 1 min. at 40°C, and the culture then incubated at 30°C for 20 min. Samples were then transferred to various lower temperatures and incubated with gyrotary shaking. Samples (1ml) were mixed with a few drops of toluene for measurement of β-galactosidase, and taken as described above for alkaline phosphatase. Assays were carried out as previously described (17) and rates of synthesis determined over 80 min. of incubation.

RESULTS AND DISCUSSION

Fatty-acid starvation. When thoroughly washed cells were incubated without oleate, growth continued for approximately 1½ generations and then stopped, as previously reported (24, 5). During this time alkaline phosphatase continued to be made at a rate close to that of oleate supplemented cells (Fig. 1). This result is in accord with those of Nunn and Cronan (5) who find little affect of oleate starvation on the assembly of a functional lactose permease, but contrasts with similar experiments on alkaline phosphatase synthesis (12). In this latter study, a non-leaky unsaturated fatty-acid auxotroph (ole-28) showed a negligible rate of alkaline phosphatase synthesis in the absence of oleate, compared to oleate supplemented cells. However, the cells were starved for oleate and concomitantly starved for inorganic phosphate to initiate alkaline phosphatase. Possibly the phosphate depletion is inadequate to derepress synthesis in the absence

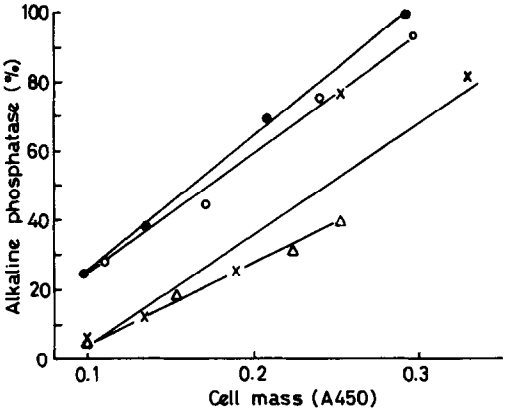

Fig. 1 Effect of oleate starvation on synthesis of alkaline phosphatase. See Experimental for details. Two experiments are shown:

(1) With (●) and without (○) oleate.
(2) With (X) and without (△) oleate.

of oleate; alternatively, the combined effects of phosphate depletion and oleate starvation may have (unknown) consequences which oleate starvation alone does not have. Silbert and Schlesinger have also found no effect of oleate starvation on constitutive synthesis of alkaline phosphatase (quoted in 3).

Fatty-acid replacement. We have compared the rates of synthesis of β-galactosidase and alkaline phosphatase at 30°C and below, using cells grown with palmitoleate and palmitelaidate (Figs. 2 and 3). We find that in palmitelaidate grown cells, in contrast to palmitoleate grown cells, the rates of synthesis of both alkaline phosphatase and of β-galactosidase decrease sharply below 27-30°C. The onset of the phase transition from a fluid to a gel-like state in membranes from E. coli grown with palmitelaidate occurs at 33°C, the mid-point of the transition occurring at 27°C (9); a discontinuity of the Arrhenius plot for β-galactoside transport occurs at about 30°C (20,9). Our results are generally consistent with these values. The effect on protein synthesis is consistent with the reported effects on

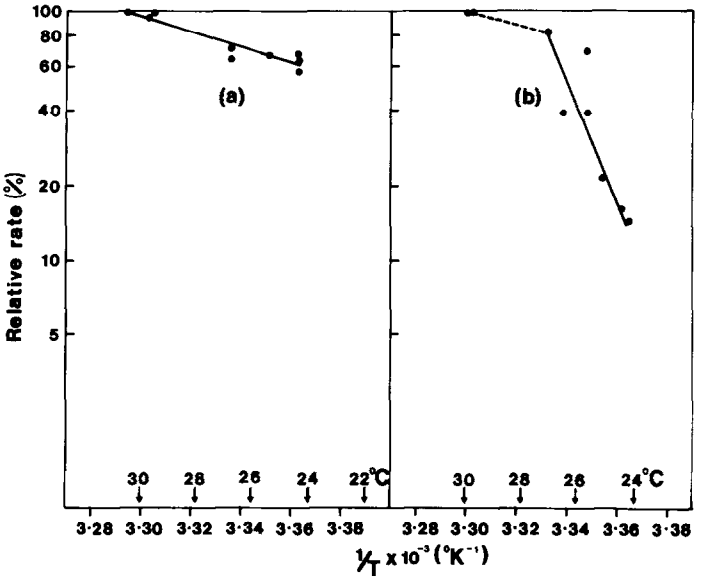

Fig. 2 Arrhenius plots of relative rate of alkaline phosphatase synthesis. (a) Cells grown with palmitoleic acid. (b) Cells grown with palmitelaidic acid.

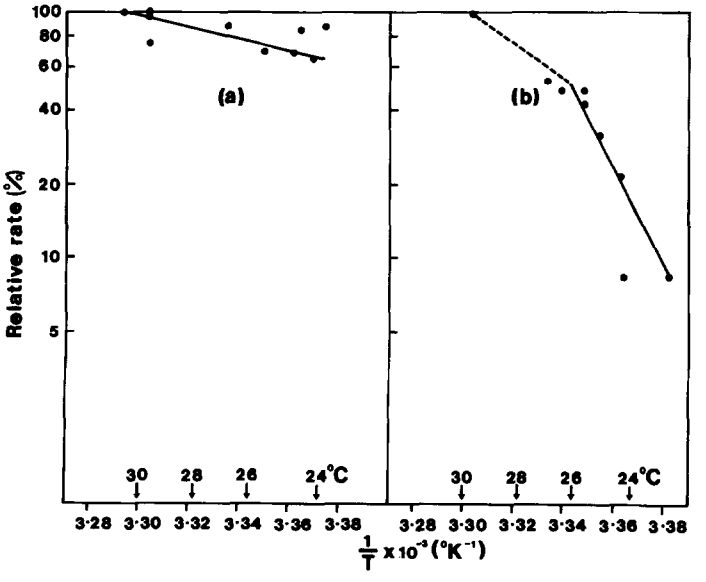

Fig. 3 Arrhenius plots of relative rate of β-galactosidase synthesis. (a) Cells grown with palmitoleic acid. (b) Cells grown with palmitelaidic acid.

growth and respiration (19). Kimura and Izui have reported similar experiments, but using phosphate starvation to initiate alkaline phosphatase synthesis; they reported that alkaline phosphatase synthesis was much more sensitive to a reduction in temperature than bulk protein synthesis (13). No definitive explanation for the difference between our results and those of Kimura and Izui is apparent, but the reason is possibly related to their use of phosphate starvation.

Despite the reduced protein synthesis in palmitelaidate grown cells below 27-30°C, a preferential effect on alkaline phosphatase could presumably have been observed. That it was not observed may reflect the fact that during secretion, the protein is thought to be in an extended conformation (28-32) and hence relatively insensitive to membrane fluidity.

ACKNOWLEDGEMENT

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