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THE LOSS OF *MESO*-TARTRATE DEHYDRATASE ACTIVITY FROM INDUCED CELLS OF PSEUDOMONADS

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SUMMARY

1. *Meso*-tartrate-grown pseudomonads rapidly lost the induced enzyme, *meso*-tartrate dehydratase, in the absence of the inducer. The rate of enzyme loss was similar in washed cells resuspended without a carbon source and in cells continuing to grow in succinate or glucose media. Azide or dinitrophenol did not affect the rate of enzyme disappearance. The level of another inducible enzyme, histidine ammonia-lyase, did not drop under these conditions.

2. Enzyme destruction in whole cells was markedly dependent on temperature, being 7% per 30 min at 25° and almost 10 times as fast at 37°. Temperatures of 25° or higher also inactivated the enzyme in extracts. In extracts, addition of tartrate isomers or incubation under N_2 stabilized the dehydratase, whereas glucose or succinate had no effect. These findings could be most readily explained by assuming that *meso*-tartrate dehydratase is a heat-sensitive protein which can be protected against denaturation *in vivo* by the inducer and its analogues or by incubation under N_2.

INTRODUCTION

Turnover measurements have shown that proteins are not degraded in growing microbial cells and that their breakdown does not exceed 10% per h in starved cells[1]. Additional proof that most microbial proteins are stable comes from measurements of inducible and repressible enzymes in cells transferred from conditions of derepression to those of repression. In such experiments, the total enzyme activity usually remains constant and specific activity falls in step with continued protein synthesis[1].

However, certain enzymes may be lost from both growing and resting cells at rates exceeding 50% per h. Among these are lysine decarboxylase[2] and RNA polymerase[3] in *Escherichia coli*, glycerol dehydrogenase[4] in *Aerobacter aerogenes*, α-isopropylmalate synthetase and isopropylmalate isomerase[5] in *Salmonella typhimurium*, α-glucosidase[6] in yeast, UDP-galactose polysaccharide transferase[7] in *Dictyo-*

stelium discoideium and acid phosphatase[8] in Euglena spec. Similar losses of particular enzymes occur in plant and animal cells[9,10]. With the exception of lysine decarboxylase[2], the mechanisms underlying these specific losses of enzyme activity are not clear.

Soil pseudomonads metabolize *meso*-tartrate through an inducible enzyme, *meso*-tartrate dehydratase, which catalyses the conversion of tartrate to oxaloacetate[11]. The present work describes the loss of preformed *meso*-tartrate dehydratase in two strains of pseudomonads, its kinetics at different temperatures and the factors stabilizing the enzyme in whole cells and in extracts.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade with the exception of *meso*-tartaric acid (purified grade, Calbiochem) and (−)-tartaric acid (Grade A, Calbiochem).

Bacterial strains

Pseudomonas md9, metabolizing all the three isomers of tartaric acid, and Pseudomonas m_1, utilizing the *meso*- but not the (+)- or (−)-isomers, are fluorescent soil pseudomonads. Both strains have been described previously[11].

Growth and harvest of cells

Cells were grown in a basal medium containing % (w/v):$(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7\,H_2O$, 0.05; $CaCl_2$, 0.005; $Fe(NH_4)_2(SO_4) \cdot 6\,H_2O$, 0.0001; yeast extract (Difco), 0.0025; Sorensen phosphate buffer, 0.007 M; final pH 7.2. Carbon sources were added to the basal medium to a final concentration of 0.1–0.4% (w/v).

Cultures were incubated in erlenmeyer flasks with shaking on a New Brunswick rotary shaker Model V or in a New Brunswick Model RW 650 shaking water bath at 33° unless otherwise stated. The ratio of flask to medium volume was 5:1. Cultures were centrifuged at 5000 × *g* at room temperature, the cells were washed once with distilled water or Sorensen phosphate buffer (pH 7.2) and resuspended in the desired medium or used for the preparation of cell-free extracts. For resuspension in medium without carbon source, the basal medium described above was used. Bacterial dry weight was determined from a calibration curve of turbidity (Klett–Summerson Photometer, Filter 42) against dry weight.

Preparation of extracts

Cell-free extracts were obtained by crushing frozen cells in a Hughes press or by breaking in a 10KC Raytheon Sonic Oscillator. Both methods yielded extracts with similar specific activities of *meso*-tartrate dehydratase. After being crushed in the Hughes press, cells were resuspended in 0.02 M Sorensen phosphate buffer (pH 7.2), sonicated for 20 sec to reduce viscosity, and centrifuged at 17 000 × *g* for 20 min at 4°. For sonication, cells were suspended in 0.02 M phosphate buffer (pH 7.2), sonicated for 10–15 min and centrifuged at 17 000 × *g* in the cold. The supernatants were dispensed into small volumes and stored at −20°, since repeated thawing and freezing led to loss of dehydratase activity.

Assay of meso-tartrate dehydratase

Enzyme assays on whole cells were performed as previously described[12] with the substitution of 0.05 M Tris buffer (pH 8.2) for the phosphate buffer used earlier. The higher pH obtained was still in the pH optimum range for dehydratase activity and gave more rapid lysis of cells in the lysozyme–EDTA mixture. Samples from cultures were prepared for assay by centrifuging, washing once and resuspending to a known turbidity. Cells suspended in basal medium without carbon source could be assayed by transferring samples directly to the assay mixture provided the final EDTA concentration was raised from 0.004 M to 0.008 M. This technique was used whenever cells in basal medium were examined.

Enzyme in extracts was assayed by incubating in a mixture of 0.05 M Tris, 0.05 M *meso*-tartrate and 0.04 M EDTA, (final pH 8.2) for 10 min at 30°. The reaction was stopped with trichloroacetic acid and the accumulated keto-acids were determined by the method of FRIEDEMANN AND HAUGEN[13]. A dehydratase unit was defined as the amount of enzyme which formed 1 μmole oxaloacetate from *meso*-tartrate per min at 30°.

Assay of histidine ammonia-lyase

Extracts were prepared by sonication and the enzyme assayed by the method of TABOR AND MEHLER[14]. An enzyme unit is defined as the amount forming 1 μmole urocanic acid per min at 30°.

Estimation of tartaric acid

Tartaric acids were estimated in culture supernatants by a colorimetric method based on the incubation of tartrate isomers with ferrous ions followed by treatment with 2,4-dinitrophenylhydrazine and NaOH (ref. 15).

RESULTS

Meso-tartrate dehydratase loss from whole cells

Growth on *meso*-tartrate induces formation of a specific *meso*-tartrate dehydratase in strains m_1 and md-9 (ref. 11). *Meso*-tartrate-grown cells of both strains lost this enzyme activity when they were washed and reincubated in basal medium containing no carbon source or when they entered the stationary phase of growth after

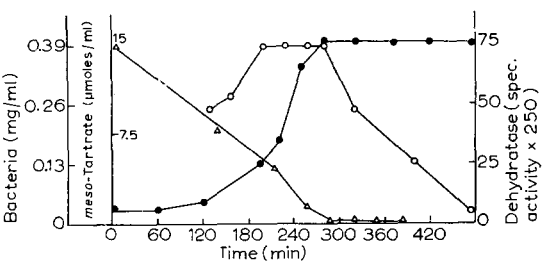

Fig. 1. Loss of *meso*-tartrate dehydratase activity by m_1 during the stationary phase of growth. Kinetics in culture shaken at 33°. ●—●, bacterial dry weight; ○—○, dehydratase specific activity; △—△, *meso*-tartrate in medium.

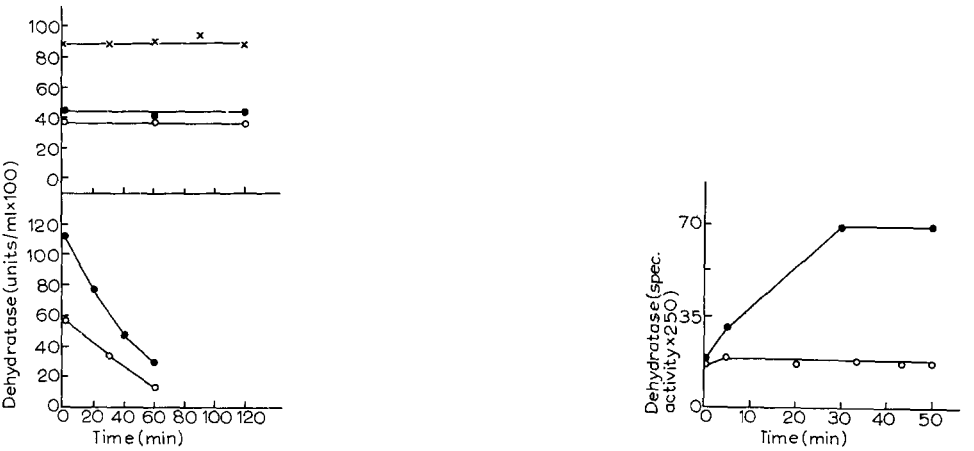

Fig. 2. Loss of dehydratase by md-9 in the presence of various carbon sources. Washed *meso*-tartrate-grown cells were resuspended in basal medium *plus* 0.4% (w/v) of carbon source and shaken at 33°. Upper graph: ×—×, *meso*-tartrate *plus* 100 μg/ml chloramphenicol; ●—●, (+)-tartrate; ○—○, (−)-tartrate. Lower graph: ●—●, succinate; ○—○, glucose.

Fig. 3. Resynthesis of *meso*-tartrate dehydratase after deadaptation. After 3 h in the stationary phase cells of m_1 were centrifuged, washed and resuspended in *meso*-tartrate medium with and without chloramphenicol at 33°. ●—●, no chloramphenicol; ○—○, 100 μg chloramphenicol/ml.

utilizing all the *meso*-tartrate (Fig. 1). Chloramphenicol (100 μg/ml), sodium azide (10^{-4} M) or dinitrophenol (10^{-3} M) did not affect the rate of enzyme loss. The dehydratases of the two strains behaved identically in these and all subsequent experiments.

Dehydratase disappearance did not depend on the absence of a carbon source since cells lost enzyme at a similar rate after washing and transfer to a glucose or succinate medium (Fig. 2). In contrast, the dehydratase level did not fall when growth continued in (+)- or (−)-tartrate media nor did the activity drop in the presence of *meso*-tartrate and chloramphenicol to prevent further induction (Fig. 2). The kinetics of enzyme loss and stabilisation were similar whether cells were resuspended with these carbon sources in complete medium or in phosphate buffer not supporting cell growth.

The fall in dehydratase activity could be due to the loss of an activator or to the accumulation of an inhibitor. Cells from the logarithmic phase were lysed with EDTA and lysozyme and the lysates mixed with lysates from cells harvested in the stationary phase after enzyme loss. The total activity of the combined lysates was the sum of the individual activities; thus no indication was obtained of a role played by activators or inhibitors. Glutathione, cysteine or 2-mercaptoethanol did not restore activity to extracts from deadapted cells. Finally, deadapted cells reformed enzyme only by protein synthesis *de novo*, chloramphenicol preventing reinduction by *meso*-tartrate (Fig. 3).

We found that instability, as shown by *meso*-tartrate dehydratase, is not a general property of inducible enzymes in md-9. This organism formed (−)-histidine ammonia-lyase (EC 4.3.1.3) when grown with histidine as the sole carbon source and cells retained their original level of this enzyme (53 munits/mg protein) during 2 h incubation in a medium without carbon source.

Effect of temperature and anaerobiosis

Temperature markedly influenced the rate at which washed cells lost dehydratase (Fig. 4). Growing cells at one temperature and reincubating the washed cells at another showed that only the temperature during reincubation influenced the rate. At 37° activity was lost almost 10 times faster than at 25°, a temperature dependence normally associated with polymer denaturations rather than enzymatic

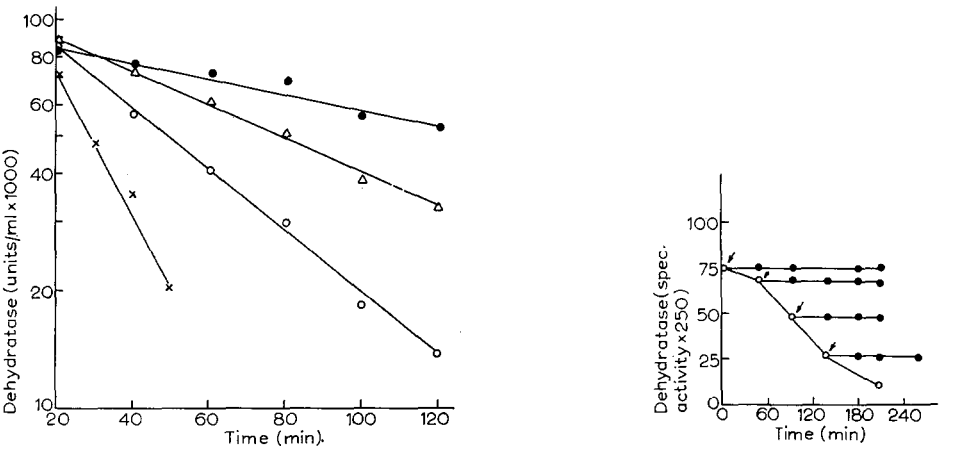

Fig. 4. Effect of temperature on dehydratase loss from whole cells. *Meso*-tartrate-grown cells of md-9 were centrifuged, washed and shaken in basal medium at various temperatures. ●—●, 25°; △—△, 30°; ○—○, 33°; ×—×, 37°.

Fig. 5. Effect of anaerobiosis on dehydratase loss. Cells of m_1 grown on *meso*-tartrate were transferred at various stages, indicated by arrows, of the stationary phase to incubation under N_2 at 33°. ○—○, air; ●—●, N_2.

reactions. Strain md-9 grows well on various carbon compounds, including *meso*-tartrate, between 20° and 37°. The temperatures inactivating the dehydratase are therefore physiological.

All three tartrate isomers prevented the loss of dehydratase at 30° or 37° as they did at 33° (Fig. 2) and cells growing logarithmically on *meso*-tartrate between 25° and 37° contained comparable amounts of dehydratase (enzyme units/mg protein: 25°, 0.56; 30°, 0.73; 33°, 0.69; 37°, 0.49).

TABLE I

EFFECT OF TEMPERATURE ON DEHYDRATASE ACTIVITY IN EXTRACTS OF md-9

Extract of *meso*-tartrate grown cells was diluted with 0.05 M Tris buffer (pH 7.6) to contain 0.94 mg protein/ml and incubated without shaking.

Incubation time (min)	*% loss of activity at*				
	0°	*25°*	*30°*	*33°*	*37°*
7	1	16	32	41	55
15	7	21	52	60	73

Incubation in a N_2 atmosphere stopped the loss of dehydratase by washed cells or by stationary phase cells at any stage of the deadaptation process (Fig. 5).

Meso-tartrate dehydratase inactivation in extracts

The dehydratase activity of extracts dropped markedly when these were exposed to temperatures above 25° for short periods (Table I). As may be expected, the absolute rates of inactivation varied with the protein concentration in a particular extract. In contrast, histidine ammonia-lyase in an extract of histidine-grown cells

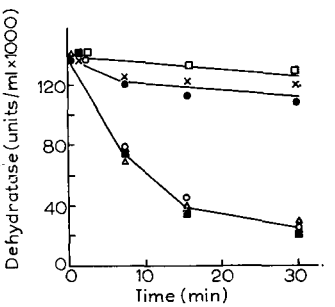

Fig. 6. Effect of substrate addition or anaerobiosis on dehydratase activity in extracts of md-9. Extract was diluted with 0.05 M Tris (pH 7.6) to give a final protein concentration of 0.82 mg/ml. Neutralised solutions of the various carbon compounds were added to give a final concentration of 1 mM. Anaerobic conditions were obtained by flushing a test tube for 5 min at 0° with N_2 and stoppering. ■—■, no addition; ○—○, succinate; △—△, glucose; ●—●, (+)-tartrate; □—□, no addition, N_2; ×—×, (−)-tartrate.

dropped from a level of 69 munits/mg protein to 61 munits after 90 min incubation at 37°.

The addition of (+)- or (−)-tartrate or incubation under N_2, factors that prevent enzyme loss in whole cells, also protected the enzyme from inactivation in extracts (Fig. 6). *Meso*-tartrate could not be tested since its rapid metabolism to ketoacids interfered with subsequent enzyme assays. Glucose or succinate, which did not stabilize the enzyme in whole cells, also had no effect on the rate of inactivation in extracts (Fig. 6).

DISCUSSION

Three factors governed the rate at which *meso*-tartrate dehydratase activity was lost in both whole cells and extracts. These were the addition of the substrate or its stereoisomers, the presence of oxygen and the temperature of incubation.

The marked temperature dependence of dehydratase inactivation, both in extracts and in whole cells, indicates a denaturation rather than an enzyme-catalysed process. Oxygen appears to participate directly in the reactions leading to enzyme loss since incubation under N_2 stabilized the activity in extracts as well as in whole cells. It may be relevant that a tartrate dehydratase from a different strain of Pseudomonas is built of polypeptide subunits[16]. Denaturation of this enzyme into

its subunits led to complete loss of activity, and repolymerization could be obtained only under strongly reducing conditions.

Under aerobic conditions, *meso*-tartrate dehydratase was markedly sensitive to those temperatures that are optimal for the growth of the pseudomonads. This sensitivity, whatever its molecular basis, would provide the simplest explanation for the observed losses *in vivo*. In this interpretation, *meso*-tartrate would stabilise the dehydratase through the well-known protective effect of substrates against enzyme denaturation by various agents, including heat. Mutations resulting in increased heat-sensitivity of a particular enzyme are well known[17] and such mutants lose preformed enzyme when incubated at physiological temperatures. In a natural habitat, a heat-sensitive inducible enzyme should not place the cell at a selective disadvantage if the inducer stabilises the protein.

Meso-tartrate dehydratase loss appears to be simpler and possibly quite different from several other deadaptations. Loss of glycerol dehydrogenase in *A. aerogenes*[4], of α-glucosidase[6] and galactozymase[18] in yeast and of thymidylate kinase[9] in Lillium spec. requires the addition of an exogenous carbon source and is inhibited by dinitrophenol or azide. The acid phosphatase[8] of Euglena spec. is destroyed only when inorganic phosphate is added to cultures. In contrast, *meso*-tartrate dehydratase was lost whenever the inducer was removed and there was no dependence on active catabolism or aerobic energy supply.

Acid phosphatase[8], glycerol dehydrogenase[19] and α-glucosidase[6] are stable in extracts at temperatures leading to rapid loss in whole cells. Though this may indicate that there is no relation between heat denaturation and enzyme loss *in vivo*, results obtained by BENNUM AND BLUM[8] and by GEST AND MANDELSTAM[20] justify a further discussion of the possibility of such a relationship.

Acid phosphatase loss from whole cells of Euglena spec., like the loss of *meso*-tartrate dehydratase, is markedly affected by temperatures in the physiological range for growth[8]. The purified phosphatase, however is heat-stable. One interpretation would be that on readdition of inorganic phosphate, leading to enzyme loss in whole cells, an intermediate is formed which renders the enzyme heat-sensitive. This would be analogous to the heat-lability of β-galactosidase in the presence of fructose diphosphate[20]. GEST AND MANDELSTAM[20] have suggested that the combination of enzymes with catabolites other than their substrates may be a general phenomenon related to catabolite repression. The formation of an enzyme–catabolite complex would not necessarily lead to thermolability of the protein, but where it does, loss of preformed enzyme may result. Such a scheme would explain the need for active carbon metabolism which is a feature of many deadaptations. It would also provide a basis for the specificity of enzyme loss, not easily explained on the known behaviour of proteolytic enzymes.

In this view, both the loss of *meso*-tartrate dehydratase and of the enzymes in the deadaptations discussed above would be due to the instability of the proteins at the incubation temperature. The dehydratase, however, would be a naturally heat-sensitive protein whereas the other enzymes are rendered heat-sensitive under particular conditions.

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