Thermal Inactivation and Reactivation of an Enzyme *in vivo* PANTOTHENATE HYDROLASE OF *PSEUDOMONAS FLUORESCENS*

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Thermal inactivation and reactivation of pantothenate hydrolase were studied in whole cells of *Pseudomonas fluorescens*. The enzyme is susceptible to thermal inactivation in whole cells at 37–40°C, and is reactivated when the temperature is lowered again. Chloramphenicol does not prevent reactivation. The activation energy of enzyme inactivation *in vivo* is about 540 kJ/mol. This activation energy is 220 kJ/mol *in vitro*, but it is increased to 550–630 kJ/mol by several metabolites, such as succinate, glyoxylate and oxalate. Generally, good carbon sources, causing rapid growth, protect the enzyme from thermal inactivation *in vivo*, and enable reactivation to occur at a fast rate. The enzyme is also inactivated below 35°C, showing an activation energy of about 35 kJ/mol. Good carbon sources prevent this inactivation as well, and cause slight reactivation. Glycine, although not utilized for growth, protects the enzyme well from this inactivation but not from inactivation at 37–40°C, and prevents reactivation totally. From the activation energies of inactivation and the effects of the various carbon sources, it appears possible that changes in the concentrations of intracellular metabolites may be responsible for the changes in inactivation and reactivation.

Although many enzymes can be reactivated after thermal inactivation *in vitro* (Swartz *et al.*, 1958; Zimmerman & Coleman, 1971; Tanford, 1968), this property has very rarely been observed *in vivo*. Johnson *et al.* (1942) suggested that reversible denaturation is 'a basic mechanism in the biological effects of temperature', and reported that the decrease in the luminescence of luminous bacteria with increasing temperature was due to reversible denaturation of luciferase.

Another example of a reversible, thermally induced conformational change *in vivo* has been published by Ron & Shani (1971), who suggest that homoserine trans-succinylase (*O*-succinyl homoserine synthetase) is partly inactivated in *Escherichia coli* at 42–47°C. This inactivation seems to be the cause of the cessation of growth at this temperature.

In the present paper I describe thermally induced inactivation and reactivation of pantothenate hydrolase in *Pseudomonas fluorescens*, and show how this reversible inactivation is affected by growth conditions. The enzyme is the first one in the pathway for degradation of pantothenate, which has been extensively studied in this laboratory (Nurmikko *et al.*, 1966, 1971; Mäntsälä & Nurmikko, 1970; Mäntsälä, 1971).

Materials and Methods

Bacterial strain and media

In this work I have used the bacterium *Pseudomonas fluorescens* strain UK-1, which has been

isolated from sea water. This bacterium shows a diauxic growth curve on pantothenate. The cells were harvested during the exponential growth in the first, β -alanine-dependent, growth phase. The medium was: 10 mM carbon source, 20 mM-KH₂PO₄, 5 mM-(NH₄)₂SO₄, 1 mM-MgSO₄ and 10 μ M-FeSO₄. The pH was adjusted to 7.0 with 5M-KOH. In this paper the media are named according to the carbon source, and 'salt solution' means the medium without any carbon source.

Cells from a pantothenate-agar culture were grown in 100 ml of pantothenate medium and transferred to 1.5 litres of the same medium. The culture was aerated with an air flow of about 1.5 litres/min per litre of medium, and the temperature was 25°C. The cells were harvested by centrifugation and stored in fresh pantothenate medium at 2°C.

Reagents

Calcium pantothenate was from Fluka AG, Buchs, Switzerland and of Purum Grade. The calcium salt was converted into the potassium salt by precipitating the Ca²⁺ with K₂CO₃ and filtering. For enzyme assays Ca²⁺ was removed from pantothenate with an ion-exchanger (Dowex 50), which simultaneously removed β -alanine (present as an impurity). The radioactive pantothenate was from New England Nuclear, 60772 Dreieichenhain, Germany, and the radioactive methionine from The Radiochemical Centre, Amersham, Bucks., U.K. Chloramphenicol was purchased from Parke, Davis and Co. Ltd., Greenford, Middx., U.K. 2,5-Diphenyloxazole and

p-bis-(*O*-methylstyryl)benzene were from Packard, Downers Grove, Ill. 60515, U.S.A.

Enzyme assay

The activity of pantothenate hydrolase was determined with [$1\text{-}^{14}\text{C}$]pantothenate. The radioactive β -alanine formed was measured. The method is based on that of Mäntsälä & Nurmikko (1970).

The cells were centrifuged at 9000*g* for 5 min from 300 μl samples (20–60 μg dry wt.) of the cell suspension; the supernatant was removed by suction and the samples of packed cells were stored at -20°C . The cells were suspended in 50 μl of 5 mM-potassium phosphate, pH 7.7, and disrupted by freezing and thawing four times (-40°C , ethanol bath; $+20^\circ\text{C}$, water bath). Before the last thawing 50 μl of the following solution was added: 90 mM-potassium [$1\text{-}^{14}\text{C}$]pantothenate (30000 c.p.m./50 μl)–60 mM-potassium phosphate, pH 7.7. The tubes were then placed in a water bath at 25°C . The total volume of the reaction mixture was thus 100 μl . After incubation for 30 min the reaction was stopped by heating for 1.5 min at 100°C . Samples (25 μl) were pipetted on to Whatman no. 1 chromatography paper and the chromatograms were developed in butan-1-ol–acetic acid–water (25:4:10, by vol.). The β -alanine spots (R_F 0.28) were cut off, dipped into scintillation liquid and counted for radioactivity with a liquid-scintillation counter.

Rate of protein synthesis

The rate of protein synthesis was measured as incorporation of radioactive methionine into proteins. Samples (10 ml) were taken out of the culture and 100 μl of 1 mM-L-[^{35}S]methionine (100000 c.p.m./100 μl) was added. Final concentration of methionine was then 10 μM . After incubation for 15 min at 25°C 1.2 ml of 90% (w/w) trichloroacetic acid was added. After 10 min the precipitated matter was filtered off, by using Whatman GF/C glass-fibre paper, and washed with about 15 ml of 5% (w/w) trichloroacetic acid containing 1 mg of methionine/ml, and finally with about 5 ml of acetone. The dry filter-paper discs were dipped into scintillation liquid and counted for radioactivity.

Measurement of radioactivity

All radioactivity counting was carried out in a liquid-scintillation counter (Wallac Decem-NTL³¹⁴). The scintillation liquid contained 4 g of 2,5-diphenyl-oxazole and 0.1 g of *p*-bis-(*O*-methylstyryl)benzene in 1 litre of toluene.

Heat-treatment

The cells were harvested from pantothenate medium by centrifugation, washed once with salt

solution and suspended in 150 ml of medium. Before heat-treatment the culture was incubated for 1 h at 25°C , after which it was transferred for 1 h to the water bath at 41°C (the temperature in the flask was then $40 \pm 0.2^\circ\text{C}$) and returned after this treatment to the water bath at 25°C . The culture was aerated all the time. The temperature rose to 40°C in about 10 min and decreased again to 25°C in 10 min.

Results

Inactivation of pantothenate hydrolase

Pantothenate hydrolase is a very thermolabile enzyme. It is inactivated even at 28°C . Fig. 1 shows the Arrhenius plots of the inactivation of the enzyme in cell extracts in phosphate buffer, either alone or combined with some metabolites. The metabolites were chosen for their protective effect from a large number of tested compounds. Fig. 1 shows that they increase the activation energy of the inactivation

Fig. 1. Arrhenius plots of the inactivation of pantothenate hydrolase *in vitro*

The enzyme solutions in 20 mM-potassium phosphate buffer, pH 7.7, were kept at different temperatures and samples for enzyme assay were taken at 5 min intervals. The values of k , the velocity constant, were calculated as an average of k for three successive 5 min periods. The added metabolites were each at 5 mM: ○, phosphate; □, succinate; △, glyoxylate; ●, oxalate.

Fig. 2. Arrhenius plots of the inactivation of pantothenate hydrolase in whole cells

The cells were kept in salt solution at 25°C for 1 h and then at different temperatures. The samples for enzyme assay were taken at intervals of 10 min. The values of k were calculated as an average of k for three successive 10 min periods.

nearly threefold. Without them the value is 220 kJ/mol, and with succinate it is 570, with glyoxylate 630 and with oxalate 610 kJ/mol. A shift to the left of the graphs means a protective effect. Oxalate is the best protector. An inactivation rate of 5%/min is reached with oxalate at a temperature 7°C higher than with phosphate.

In Fig. 2 the inactivation rates are shown in whole cells without a carbon source. These show the existence of at least two types of inactivation. Below 35°C the activation energy is about 35 kJ/mol, as in normal biological processes, but between 37° and 40°C it is about 550 kJ/mol. Such high values of activation energy are typical of denaturation or other conformational changes (Joly, 1965). Other measurements in the latter region have led to the value 920 kJ/mol, although it is impossible to give exact values when the other events in the cell at this temperature no doubt also affect the values. However, the order of magnitude is the same as with the best protectors *in vitro*, and moreover, inactivation occurs in the same temperature range *in vivo* and *in vitro*.

Reactivation of the enzyme *in vivo*

After pantothenate hydrolase is inactivated in whole cells of *P. fluorescens* by heat-treatment for 1 h at 40°C, the activity can be restored by lowering the temperature and adding a suitable carbon source (Fig. 3). Inhibition of protein synthesis does not prevent the increase of enzyme activity, so that the phenomenon cannot be due to synthesis of new enzyme. However, when the inhibitor is added immediately after the heat-treatment, it slows down reactivation. This may be explained by assuming that for optimum activation

Fig. 3. Reactivation of pantothenate hydrolase after thermal inactivation

Cells grown on pantothenate were kept in salt solution at 25°C for 1 h, and then transferred to 40°C and after 1 h returned to 25°C. (a) Glutamate (5mM) was added at the end of the heat-treatment (stippled area) and chloramphenicol (arrows, 50 µg/ml) in (b) at the same time as glutamate and, in (c) 0.5h, later. o, Enzyme activity; ●, methionine incorporation into proteins; Δ, turbidity of the culture (Klett units, with filter no. 62);

Fig. 4. Arrhenius plot of the reactivation of pantothenate hydrolase in whole cells

Inactivation and reactivation were carried out as described in Fig. 3(a) except that the reactivation temperature was varied. The reactivation rates were calculated from the activity values between 0.5 and 2 h after the heat-treatment.

conditions the synthesis of some other enzyme is necessary. The measurement of the incorporation of methionine into protein shows that the rate of protein synthesis is low at the end of the heat-treatment and increases drastically when the growth is beginning. Chloramphenicol prevents the incorporation of methionine totally.

Fig. 4 presents the Arrhenius plot for reactivation of the enzyme. The values obtained show that the activation energy is low, about 40 kJ/mol, and very different from that of inactivation.

Effect of various carbon sources on inactivation and reactivation

As may be expected from the results in Fig. 1, different carbon sources also have very different effects on the rate of inactivation *in vivo*. The results in Table 1 are for carbon sources that were also present in the medium during the heat-treatment. Generally, good carbon sources, causing rapid growth, protect the enzyme well. The very good protective effect of propionate may be due to the fact that its degradation leads directly to the succinate side of the citrate cycle, perhaps causing an increase in concentrations of protective dicarboxylic acids in the cell.

Table 1. Protection of pantothenate hydrolase from heat-inactivation by various carbon sources

Cells grown on pantothenate were transferred to various carbon sources (each at 5 mM) and kept for 1 h at 25°C and then at 40°C. The samples for enzyme assay were taken at intervals of 10 min during the heat-treatment. The inactivation rates were calculated as an average of the rates for three successive 10 min periods between 10 and 40 min from the beginning of the heat-treatment.

Carbon source	Rate of inactivation (%/min)	Activity after 0.5 h at 40°C (%)
Propionate	0.56	90
Succinate	0.60	83
Glucose	0.91	72
Glutamate	1.15	78
Valine	2.0	69
Ethanolamine	6.0	31
Glycine	10.3	8
None	11.1	7

Table 2. Reactivation rates of pantothenate hydrolase on various carbon sources

Inactivation and reactivation were carried out as described in Fig. 5. The reactivation rates were calculated from the activity values between 0.5 and 2 h after the heat-treatment.

Carbon source	Reactivation rate (% of original activity/h)
Glutamate	63
4-Aminobutyrate	52
Glucose	37
Succinate	35
Ethanolamine	33
Propionate	24
Valine	24
β -Alanine	18
Ornithine	17
Serine	6
Sarcosine	5
Glyoxylate	4
Glycine	0
None	11

Fig. 5 and Table 2 show that carbon sources also affect the reactivation of the enzyme. The compounds tested have been chosen to represent various metabolic degradation pathways and various abilities of the bacterium to utilize these compounds. Particular attention was paid to the specific region in central metabolism (from pyruvate to the citric acid cycle) to

Table 3. *Inactivation rates of pantothenate hydrolase at 25°C on various carbon sources*

Cells grown on pantothenate were transferred to various carbon sources (5mM). The inactivation rates were calculated from the activity values between 2 and 4h.

Carbon source	Inactivation rate (%/h)	Lag phase (h)	Generation time (h)
Glutamate	0	1	2.0
Glucose	0	2	2.6
β -Alanine	0	1	3.5
Succinate	1.5	1	2.6
Propionate	1	1	4.7
Ornithine	1.5	4	6.1
Valine	15	6	
Ethanolamine	38	1	17
Lysine	38	>7	
Glycine	0	>7	
Serine	1.5	6	
Glyoxylate	2	>7	
Sarcosine	3.5	>7	
None	42

Fig. 5. *Reactivation of pantothenate hydrolase by various carbon sources*

Cells were kept at 25°C for 1 h, and then at 40°C for 1 h and thereafter at 25°C. The carbon sources (5mM) were added to the medium at the end of the heat-treatment (stippled areas): (a) glutamate; (b) glucose; (c) propionate; (d) ethanolamine; (e) glycine; (f) none. ○, Enzyme activity; ●, turbidity of the cell suspension (Klett units, with filter no. 62).

which the degradation of the compounds is leading. Thus 4-aminobutyrate, valine and propionate are metabolized to the succinate side of the citrate cycle, glutamate to 2-oxoglutarate and β -alanine to acetyl-CoA. When the bacterium grows on pantothenate, β -alanine is used during the first phase of diauxic growth. Thus the flow of carbon atoms goes through acetyl-CoA. The generation time on some carbon sources and the length of the lag phase after growth on pantothenate are presented in Table 3. Generally the compounds causing the shortest lag and the shortest generation time cause the highest reactivation rates (Table 2). Reactivation occurs even without any carbon source, but glycine prevents reactivation. β -Alanine causes only a low reactivation rate. The effects of various carbon sources indicate that the presence of suitable metabolites in the cell is also important during activation. In spite of extensive experiments I have not so far succeeded in finding activating compounds or conditions leading to activation *in vitro*.

Inactivation at growth temperatures

As Fig. 2 shows, the inactivation of the enzyme below 35°C is at least partly a different phenomenon

from that between 35° and 40°C. In addition to inactivation, reactivation and proteolysis can change the activity of the enzyme. The results in Fig. 6 and Table 3 are for cells grown on pantothenate and then kept on various carbon sources at 25°C. In salt solution we find a decrease of about 40%/h in the amount of pantothenate hydrolase. The carbon sources decrease the fall and on very good carbon sources, causing rapid growth without a long lag phase, the activity of the enzyme does not decrease at all.

Fig. 7 sheds light on the relations between inactivation and decomposition. When glutamate is added to the cells in salt solution, the pantothenate hydrolase activity rises. The increase is small and never exceeds 15% of the original enzyme activity. This suggests that all inactivated enzyme has not been decomposed in proteolysis.

In the effect of chloramphenicol (Fig. 8) configuration is seen of the fact, often noted previously, that inhibition of protein synthesis also stops proteolysis (Mandelstam, 1958; Urba, 1959; Schlessinger & Ben-Hamida, 1966). Here it is seen at the level of an individual enzyme that in salt solution the decrease of the enzyme activity is smaller in the presence of chloramphenicol, and even valine with chloramphenicol is enough to stop the decrease, although on it the

Fig. 6. Inactivation of pantothenate hydrolase at 25°C by various carbon sources

Cells grown on pantothenate were transferred to various carbon sources (5mM): (a) glutamate; (b) glucose; (c) propionate; (d) ethanolamine; (e) glycine; (f) none. o, Enzyme activity; ●, turbidity of the cell suspension (Klett units, with filter no. 62).

Fig. 7. Increase in pantothenate hydrolase activity after addition of carbon sources

Cells grown on pantothenate were kept for 3h in salt solution at 25°C and thereafter glutamate (a) or β -alanine (b), both 2mM, were added (indicated by arrows). o, Enzyme activity; ●, turbidity of the cell suspension (Klett units, with filter no. 62).

Fig. 8. Effect of inhibition of protein synthesis on the inactivation of pantothenate hydrolase at 25°C

Cells grown on pantothenate were transferred to salt solution. The carbon sources and the inhibitors were added as follows: (a) chloramphenicol (50 μ g/ml); (b) valine (5mM) and chloramphenicol (50 μ g/ml); (c) no carbon source or inhibitor; (d) valine (5mM). o, Enzyme activity; ●, turbidity of the cell suspension (Klett units, with filter no. 62).

bacterium has normally a long lag and long generation time.

Effect of glycine

Glycine presents a particular case involving both inactivation and reactivation. The bacterium grows

very poorly on glycine, but the enzyme is not inactivated during growth on it, nor is the inactivated enzyme reactivated. Table 3 shows the effects of glycine and related metabolites, glyoxylate, serine and sarcosine, on the inactivation of the enzyme. Glycine totally prevents inactivation, the others nearly totally. Table 2 shows the effects of the same

substances on reactivation, and only glycine prevents it completely. Glyoxylate, serine and sarcosine retard the rise compared with the salt solution. Glycine does not protect against heat-inactivation at 40°C (Table 1).

Discussion

Johnson *et al.* (1942) described reversible denaturation as a basic mechanism in the biological effects of temperature. This mechanism is no longer regarded as of fundamental importance, because very few enzymes have been found that renature after denaturation *in vivo* or which are inactivated below the maximum temperature of growth. The existence of such a mechanism is probably limited to the temperature region of growth stoppage, where it could be of value as a mechanism for repair of damage caused by transient elevated temperature. Such a repair mechanism may be responsible for the increase in living cells of *E. coli* in phosphate buffer after heat-treatment detected by Mukherjee & Bhattacharjee (1970). In *P. fluorescens* pantothenate hydrolase is inactivated in the same temperature region as that where growth ceases. However, this inactivation cannot be the cause of the arrest of growth, because the temperature at which growth ceases is not dependent on the presence of pantothenate hydrolase.

Fortunately, disruption of the cells stabilizes pantothenate hydrolase, so that the activity does not change during measurement. If this were not so, the rate of the conformational changes would make it impossible to detect the inactivation-reativation mechanism. This may account for the lack of examples of this mechanism: they generally remain undetected unless the inactive conformation can be stabilized.

It is a fact observed repeatedly in protein chemistry that when small molecular ligands bind to proteins, the thermal sensitivity and susceptibility to proteolysis or to denaturing agents are changed at the same time (Alexandrov, 1969; Grisolia, 1964). Usually, attachment of a ligand stabilizes the protein, and its thermostability and resistance to proteinases increases. The ligand is not necessarily a substrate or coenzyme; we have some knowledge of the protective effect of other metabolites (Moses & Sharp, 1970; Südi, 1970). The effects of various carbon sources on the inactivation and reactivation of pantothenate hydrolase may be explained by the changes in the concentrations of intracellular metabolites. On suitable carbon sources the intracellular concentrations of the metabolites are large, and these metabolites protect the enzyme by binding to it. Succinate, oxalate and glyoxylate may be such metabolites. Apparently glycine is able to bind both to the

inactive and to the active conformation, stabilizing the former and preventing proteolysis of the latter. Kay & Gronlund (1969*a,b*) have shown that in *Pseudomonas aeruginosa* nearly all amino acids, especially glutamate, alanine and glycine, decrease during carbon starvation. Our preliminary measurements of the citrate-cycle acids in *P. fluorescens* also suggest changes dependent on the carbon sources.

Mandelstam (1958) has suggested that, during the inhibition of protein synthesis, low-molecular-weight compounds which inhibit degradative processes accumulate in the cell. In the presence of chloramphenicol the concentrations of the intracellular amino acids increases about twofold (Raunio & Rosenqvist, 1970; Kay & Gronlund, 1969*a*). As stated by Pine (1967), however, proteolysis also occurs during the inhibition of protein synthesis, but it is directed more than usual to recently synthesized proteins. So the apparent inhibition of proteolysis may be brought about by a decrease in the sensitivity of individual enzymes to proteolysis caused by increased concentrations of low-molecular-weight metabolites. This explanation is especially suitable for pantothenate hydrolase, since its degradation is not prevented by chloramphenicol in the absence of carbon sources, yet the presence of a carbon source prevents it.

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References

- Alexandrov, V. Y. (1969) *Curr. Mod. Biol.* **3**, 9–19
- Grisolia, S. (1964) *Physiol. Rev.* **44**, 657–712
- Johnson, F. H., Brown, D. & Marsland, D. (1942) *Science* **95**, 200–203
- Joly, M. (1965) *A Physico-chemical Approach to the Denaturation of Proteins*, pp. 199–213, Academic Press, London and New York
- Kay, W. W. & Gronlund, A. F. (1969*a*) *J. Bacteriol.* **97**, 282–291
- Kay, W. W. & Gronlund, A. F. (1969*b*) *J. Bacteriol.* **100**, 276–282
- Mandelstam, J. (1958) *Biochem. J.* **69**, 110–119
- Mäntsälä, P. (1971) *J. Gen. Microbiol.* **67**, 239–242
- Mäntsälä, P. & Nurmikko, V. (1970) *Suom. Kemistilehti B* **43**, 414–420
- Moses, V. & Sharp, P. B. (1970) *Biochem. J.* **118**, 491–495
- Mukherjee, P. & Bhattacharjee, S. B. (1970) *J. Gen. Microbiol.* **60**, 233–238
- Nurmikko, V., Salo, E., Hakola, H., Mäkinen, K. & Snell, E. E. (1966) *Biochemistry* **5**, 399–402
- Nurmikko, V., Mäntsälä, P., Elfving, R. & Kopperoinen, M. (1971) *Suom. Kemistilehti B* **44**, 248–252
- Pine, M. J. (1967) *J. Bacteriol.* **93**, 1527–1533

- Raunio, R. & Rosenqvist, H. (1970) *Acta Chem. Scand.* **24**, 2737–2744
- Ron, E. Z. & Shani, M. (1971) *J. Bacteriol.* **107**, 397–400
- Schlessinger, D. & Ben-Hamida, F. (1966) *Biochim. Biophys. Acta* **119**, 171–182
- Südi, J. (1970) *Biochim. Biophys. Acta* **212**, 213–224
- Swartz, M. N., Kaplan, N. O. & Lamborg, M. F. (1958) *J. Biol. Chem.* **232**, 1051–1066
- Tanford, C. (1968) *Advan. Protein Chem.* **23**, 187–194
- Urba, R. C. (1959) *Biochem. J.* **71**, 513–518
- Zimmerman, S. B. & Coleman, N. F. (1971) *J. Biol. Chem.* **246**, 309–317