*Biochimica et Biophysica Acta*, 452 (1976) 201—209


BBA 67961

# ON THE PARTIAL REACTIVATION OF INACTIVATED PANTOTHENASE FROM *PSEUDOMONAS FLUORESCENS*

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(Received April 27th, 1976)

## Summary

Partial reactivation of inactivated pantothenase (pantothenate amidohydrolase, EC 3.5.1.22) from *Pseudomonas fluorescens* was studied. After partial inactivation during storing, pantothenase activity is increased by 10—40% when incubated with, for instance, oxalate, oxaloacetate or pyruvate. Reactivation proceeds slowly; with oxaloacetate the stable level of enzyme activity is attained in 20—30 min. The same compounds also cause reactivation of thermally inactivated pantothenase when partial inactivation has occurred at 28—37°C. The amount of the reactivating enzyme form is relatively greater the lower the temperature during inactivation, but it never exceeds 20% of the original amount of active enzyme. Also another, unstable form of pantothenase is formed in thermal inactivation. This form becomes inactivated in a few minutes after the heat treatment, at pH 6—8 and at temperatures between 0 and 10°C. Reactivation causes special problems in enzyme kinetic measurements; for instance, curvature is found in the lines in $K_i$ determination by the Dixon plot.

## Introduction

Normally when a thermally inactivated enzyme has been reactivated this has been carried out by moving the denaturated enzyme first into a solution of urea or guanidine hydrochloride [1]. Only in a very few cases does a thermally inactivated enzyme become reactivated by decreasing the temperature [2,3]. A nearly total reactivation of thermally inactivated pantothenase (pantothenate amidohydrolase, EC 3.5.1.22) has been demonstrated in whole cells of *Pseudomonas fluorescens*, when the temperature is lowered after the thermal treatment to the normal growth temperature of the bacterium [4]. The restoration of activity is a relatively slow process, the totally reactivated level of enzyme

Abbreviation: MOPS, *N*-morpholino-3-propan-sulphonic acid

activity is reached within 2 h after the lowering of the temperature. Rate of reactivation is dependent on the carbon source of the medium. Several recent investigators have paid attention to the responsibility of ligands for the refolding phenomenon [5,6]. The role of ligands also in the reactivation of pantothenase is suggested by the dependence of pantothenase reactivation on a carbon source as well as by the difficulty of achieving reactivation after cell disruption. The present investigation aims at studying the reactivation of pantothenase with a purified enzyme preparation. A minor part of the partially inactivated enzyme can be reactivated; some small-molecule compounds like oxaloacetate, oxalate, or pyruvate are necessary to achieve reactivation.

## Materials and Methods

*Pantothenase preparation.* Pantothenase was purified from *Ps. fluorescens* UK-1 according to the method described previously [7]. The preparative disc-gel electrophoresis was omitted from the purification process. The purity of the enzyme preparation was about 90%. The enzyme preparation was stored in ice at −20°C in 35 mM potassium phosphate pH 6.8. Before using, a small quantity of the enzyme was transferred into 5 mM $K_2SO_4$ by gel-filtration on a Sephadex G-25 (Coarse) column (1 × 40 cm).

*Chemicals.* *N*-morpholino-3-propan-sulphonic acid (MOPS) was from Serva, Heidelberg, Germany and bovine serum albumin from Sigma Chemical Co., St. Louis, Mo., USA. Potassium oxalate and sodium pyruvate were from E. Merck A.G., Darmstadt, Germany. Oxaloacetic acid was from Boehringer Mannheim GmbH, Mannheim, Germany.

*Pantothenase assay.* Pantothenase was determined as described elsewhere [8]. The reaction mixture, 125 µl in total volume, contained 25 µl of enzyme solution (120—180 µg/ml), 25 µl of buffer solution (normally 100 mM potassium phosphate), 25 µl of 150 mM potassium pantothenate, 25 µl of [1-$^{14}$C]-pantothenate solution (80 000 dpm/25 µl), and 25 µl of added compound solution or water.

*Inactivation and reactivation.* Inactivation was performed in a water bath; the small test tubes contained two components of the above reaction mixture, enzyme and buffer, 25 µl of each. When the inactivated enzyme activity level was to be measured, the inactivation was stopped, the enzyme reaction was commenced by adding the substrate, the isotope, and the 'added compound', and the test tubes were moved to 20°C. When measuring reactivation after inactivation, the 'added compound' was pipetted after the inactivation, and the test tubes were moved to a water bath at 10°C for 2 h after which the substrate and the radioactive isotope were added.

## Results

*Reactivation of pantothenase inactivated during storing*

The efforts to reactivate the inactivated pantothenase are based on the previous observation that the thermally inactivated enzyme can be reactivated in whole cells [4]. Appropriate carbon sources tend to contribute to the reactivation. If the reactivation were dependent on the presence of a certain metabo-

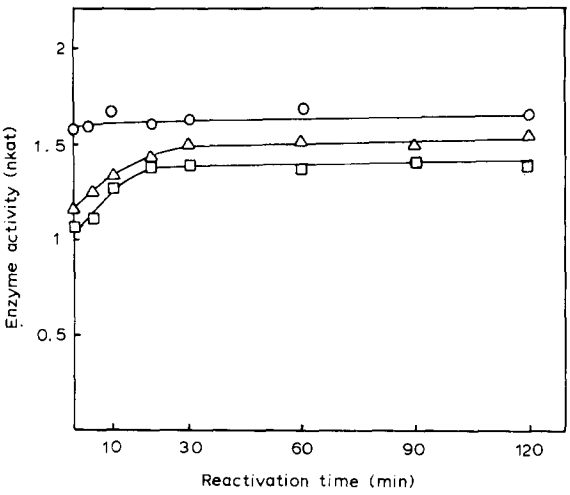


Fig. 1. Reactivation of pantothenase in the presence of oxaloacetate. Pantothenase was stored in 5 mM $K_2SO_4$ at 2°C for a week. The activity diminished during the storage to about 50% of the original. Reactivation was carried out by incubating the enzyme (40 μg/ml) in the presence of the buffer (33 mM potassium phosphate, pH 7.2) and potassium oxaloacetate. The oxaloacetate concentrations were 0 (○), 0.7 mm (△), or 1 mM (□). At various intervals samples (75 μl) were pipetted into reaction tubes containing the radiocative substrate, and the enzyme assay was accomplished as normally. (The activity in the presence of oxaloacetate is lower than in its absence due to the inhibiting effect of oxaloacetate.)

lite, reactivation might be possible also in vitro with this compound. The first positive observation indicative of this possibility is presented in Fig. 1. An increase of approx. 20—40% is obtainable upon incubating the enzyme with oxaloacetate before activity determination. The inactivation in this case occurred during storage at 2°C in 5 mM $K_2SO_4$ for a week. Table I presents the amount of the enzyme reactivated in 0.5 h in the presence of various metabolites. Oxa-

TABLE I

EFFECTS OF SOME METABOLITES ON THE REACTIVATION OF PANTOTHENASE

Pantothenase was inactivated during storage at 2°C in 5 mM $K_2SO_4$ for a week to about 50% of its original activity. The partially inactivated enzyme was incubated for 30 min at 10°C in the presence of the buffer and of various compounds, and the enzyme activities were measured. The buffer was 17 mM MOPS ($K^+$ salt) pH 6.9 and the concentrations of the tested compounds were at 1 mM, and the pantothenase amount was 17 μg/ml. Reactivation is expressed as per cent increase of the enzyme activity during the incubation.

| Compound | Reactivation (%) |
|---|---|
| Oxalate | 19 |
| Oxaloacetate | 18 |
| Pyruvate | 19 |
| Malate | 14 |
| Fumarate | 12 |
| Succinate | 10 |
| Acetate | 4 |

late, oxaloacetate, and pyruvate proved to be the most effective catalysts of reactivation.

Freezing and thawing inactivate pantothenase by approx. 45% for one freezing in 5 mM $K_2SO_4$, and by approx. 20% in 50 mM potassium phosphate pH 7.0. The activity of the enzyme thus inactivated could not be restored by incubating it with oxalate.

*Reactivation after thermal inactivation*

Slight reactivation of thermally inactivated panthothenase can be caused by oxaloacetate, 4-oxovalerate, 3-oxoglutarate and oxalate (in 8.3 mM concentrations), when the inactivation has occurred at 35° C for 30 min. The enzyme activity after inactivation is about 6%, and after subsequent reactivation 10—20% of the original activity. No compound causing inhibition or protection against thermal inactivation (Table I in ref. 9) causes total reactivation. Fig. 2 shows the effect of inactivation temperature on the reactivation between 28 and 37.1° C. At lower temperatures the proportion of the reactivated enzyme form is relatively greater, but it never exceeds 20% of the original amount of enzyme.

To increase the proportion of the reactivating enzyme form, the following changes in conditions, among others, were tested: (1) the reactivation temperatures were varied within the range 0—20° C; (2) MOPS (17 mM during reactivation) or phosphate (33 mM) was employed as buffer in inactivation and reactivation; (3) pH was varied within the range 6—9. (4) Protein addition (bovine serum albumin, 0.5%) was used to protect pantothenase during inactivation and reactivation processes; (5) Dithiothreitol (10 mM in reactivation) or mercaptoethanol (10 mM) was employed for SH-protection during inactivation

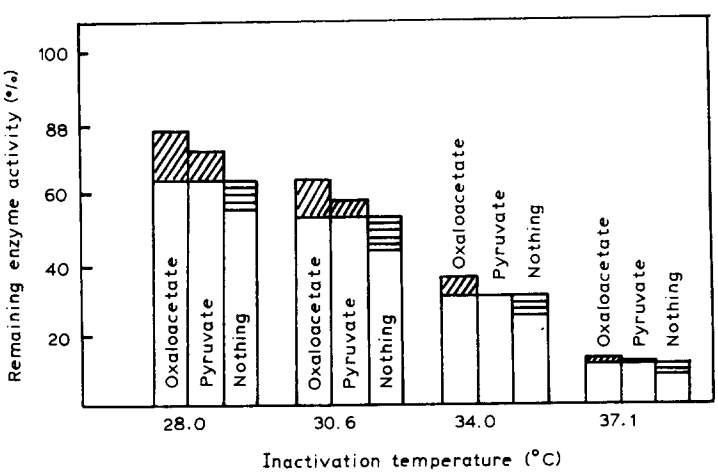


Fig. 2. Reactivation of pantothenase after inactivation at various temperatures. Inactivation was carried out in a water bath at 28, 30.6, 34.0 or 37.1°C for 10 min in test tubes containing 25 µl of the enzyme solution (180 µg/ml) and 25 µl of 50 mM MOPS ($K^+$ salt) pH 6.8, and reactivation was carried out at 10°C for 3 h; either 25 µl of 2 mM potassium oxaloacetate or 25 µl of 5 mM sodium pyruvate or 25 µl of water was added after inactivation. Enzyme activities were assayed both from uninactivated samples and after inactivation and after reactivation. □ activities after inactivation; ▨ increase in activities during reactivation; ▤ decrease in activities during 'reactivation'.

and reactivation. Under no conditions could any essential difference in reactivation be demonstrated compared to the curve described in Fig. 1.

Glycine inhibited completely the reactivation occurring after thermal inactivation in whole cells [4]. This phenomenon could not, however, be observed in vitro when reactivation occurred in the presence of 1 mM potassium oxalate and 10 mM glycine. The activity of pantothenase increased during the incubation after inactivation as well as in the presence of oxalate only.

*Rapid inactivation after thermal treatment*

Rapid inactivation below pH 7 to a somewhat lower activity level occurs if the pantothenase solution is allowed to stand at 0° C after thermal treatment. The fall of activity is almost totally accomplished during the first ten minutes after thermal treatment (Fig. 3). Under similar conditions the normal fall of enzyme activity is about 10% per 24 h. To test the possible responsibility of rapid inactivation for the development of the reactivating enzyme form, oxaloacetate (1.7 mM) was added after 2 h to the enzyme solution in an experiment like Fig. 3b. The changes in enzyme activity were observed for 2 h. It became obvious that the activity did not reach even the level established after thermal inactivation. If rapid inactivation had produced a reactivatable enzyme, the enzyme activity should have risen almost to the level achieved by reactivation immediately following thermal inactivation.

*Reactivation and enzyme kinetic measurements*

The compounds reactivating pantothenase act, simultaneously, as effective inhibitors of the enzyme reaction [9]. Reactivation seems to produce a special problem in the measurement of enzyme kinetics, and particularly inhibition kinetics. Fig. 4 presents the results obtained in a determination of the inhibition type when employing Dixon's method. Reactivation at high inhibitor concentrations contributes to the curvature of the plots. In this determination the reaction was commenced by adding the substrate to the reaction mixture to

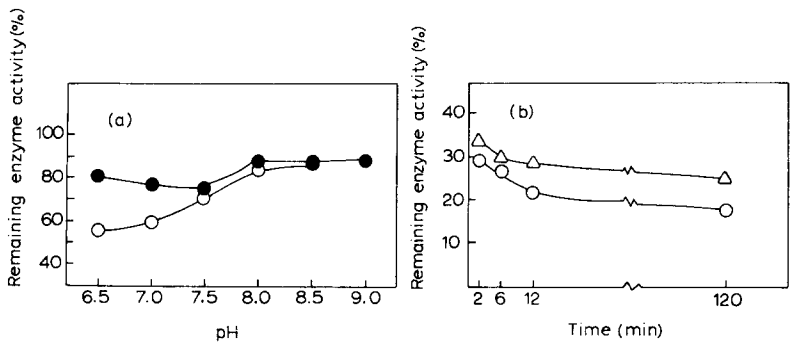


**Fig. 3. Cold inactivation of pantothenase after heat treatment. (a) Pantothenase solution (180 μg/ml) containing 5 mM potassium sulphate and 0.5 mM potassium phosphate, pH 7, was incubated at 30.6°C for 15 min. After this 25 μl of this solution was pipetted into tubes containing 25 μl of buffer (100 mM potassium phosphate of various pH values) and 25 μl of water. The tubes were allowed to stand for 2 h at 0°C. Pantothenase activities were assayed before (100% level) and after (•) the heat treatment and after standing at 0°C (○). (b) Pantothenase was inactivated for 15 min at 32.5°C in 5 mM potassium sulphate. Thereafter the inactivation mixtures were moved to water baths at 0°C (△) and 10°C (○), and samples for enzyme assays were drawn off at various intervals.**

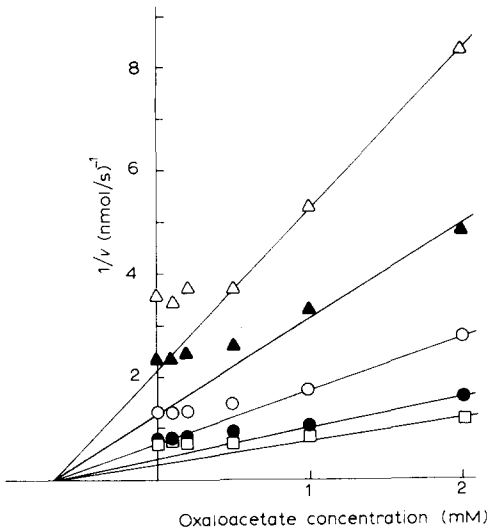


Fig. 4. Effect of reactivation on the assay of inhibition constants by the Dixon plot. Pantothenase preparation had been stored at 2°C in 5 mM potassium sulphate for some days. The assay mixture contained 25 µl of the enzyme solution, 25 µl of 100 mM potassium phosphate pH 6.6, 25 µl of the solution of potassium oxaloacetate and 50 µl of the radioactive substrate solution. The components were pipetted as follows: first oxaloacetate, then the buffer together with the enzyme, and then after about 5 min the reaction was begun with the substrate. The substrate concentrations were 30 mM (□), 20 mM (●), 10 mM (○), 5 mM (▲) and 3.3 mM (△).

which the inhibitor had been added a few minutes earlier. If the inhibitor and the substrate are added simultaneously, curvature can be almost totally avoided, and if the enzyme has been incubated with e.g. 0.2 mM oxaloacetate for several hours before the determination no curvature can be observed. Oxaloacetate concentrations, to result in reactivation needed to be above 1 mM (Fig. 4) and the $K_i$ value in the same experiment was about 0.7 mM.

## Discussion

The properties of partial pantothenase reactivation observed in the present study resemble, to some extent, the reactivation in whole cells [4]. In both cases the reactivation is rather slow, and in both cases the rate can be increased with appropriate compounds, in vivo with rapidly consumed carbon sources, and in vitro with ligands acting as inhibitors. It is observable in other proteins that the refolding of denatured proteins is slow [1,10].

The results obtained from pantothenase reactivation make it possible to describe several various enzyme conformations: native conformation ($c_n$), reactivating conformation ($c_r$), rapidly inactivating, labile conformation ($c_l$), and inactive conformation ($c_i$). $c_l$ may be identical with $c_r$, if the specific activity of $c_l$ is lower than that of $c_n$, and in reactivation $c_l$ may change into $c_n$. $c_r$ seems, however, to be more stabile than $c_l$: and in whole cells $c_r$ could not be proved to contain enzyme activity, which implies that identity is rather unlikely.

At present, several examples may be enumerated to clarify cases in which a protein denatured with urea or guanidine hydrochloride has been reactivated by removing the denaturing substance (review of Wetlaufer and Ristow [1]). Although reversibility in heat denaturation seems to be more the exception than the rule, some cases of reversible heat denaturation have been reported. Diphosphopyridine nucleotide pyrophosphatase from *Proteus vulgaris* [2], and pancreatic deoxyribonuclease [3] are subject to reactivation after thermal inactivation. In addition, the enzyme activities of homoserine transsuccinylase from *Escherichia coli* [11], of asparaginase from *E. coli* [12], and of penicillinase from *Bacillus cereus* [13] are altered by thermally induced reversible conformational changes. Several varying hypotheses have been presented about the significance of ligands in the refolding process. Their ability to effect various denaturations or renaturations remains, however, obscure. Ligands may stabilize native conformation without affecting the actual conformational change [6,14]. On the other hand, a ligand may contribute to the selection of the transition states resulting in final refolding [15], and the absence of ligands may result in wrong folding to an inactive species. Thermally denatured proteins are no random coils [10], which suggests that the presence of a ligand may stabilize some definite conformation. Then also the restoration of the native state is different in the absence and presence of the ligand. The reactivation of pantothenase by oxalate resembles the phosphofructokinase renaturation observed by Alpers et al. [15] with ATP as a catalytic factor. The ligand is not required in the enzyme reaction or the maintenance of native conformation with pantothenase, either. The same ligands are apt to inhibit enzyme reactions and to act as effective protectors against thermal inactivation [8,9]. The binding constants obtained from inhibition (0.7 mM) as well as from differential thermal inactivation (1.2 mM) are of the same order of magnitude as the effective oxaloacetate concentrations from the reactivation (Fig. 4, over 1 mM). This similarity suggests the same binding of the ligand.

The reasons for the conspicuous difference between the reactivation quantities in vivo and in vitro, have so far remained obscure. Something has evidently gone wrong at least in the refolding process since no successful reactivation could be achieved after cell disruption although the inactivation was accomplished in whole cells. It would seem rather promising to explain the differences in reactivation by the above hypothesis of Alpers et al. [15] about the directing effects of ligands. No correct ligand environment has so far been discovered in the experiments performed in vitro. Also it is possible that incorrect S-S bonds are formed in denaturation, even convenient attaching surfaces in whole cells may be of certain importance, with other proteins and cell membranes included.

## References

1 Wetlaufer, D.B. and Ristow, S. (1973) Ann. Rev. Biochem. 42, 135—158
2 Swartz, M.N., Kaplan, N.O. and Lamborg, M.F. (1958) J. Biol. Chem. 232, 1051—1066
3 Zimmerman, S.B. and Coleman, N.F. (1971) J. Biol. Chem. 246, 309—317
4 Airas, R.K. (1972) Biochem. J. 130, 111—119
5 Teipel, J.W. and Koshland, D.E. (1971) Biochemistry 10, 792—798
6 Citri, N. (1973) Adv. Enzymol. 37, 397—648

7 Airas, R.K., Hietanen, E.A. and Nurmikko, V.T. (1976) Biochem. J. 157, 409—413
8 Airas, R.K. (1976) Biochem. J. 157, 415—421
9 Airas, R.K. (1976) Biochim. Biophys. Acta 452, 193—200
10 Tanford, C. (1968) Adv. Protein Chem. 23, 122—282
11 Ron, E.Z. and Shani, M. (1971) J. Bacteriol. 107, 397—400
12 Citri, N. and Zyk, N. (1972) Biochemistry 11, 2103—2109
13 Citri, N. and Garber, N. (1963) Biochim. Biophys. Acta 67, 64—72
14 Schechter, A.N., Moravek, L. and Anfinsen, C.B. (1969) J. Biol. Chem. 244, 4981—4988
15 Alpers, J.B., Paulus, H. and Bazylewicz, G.A. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2937—2940