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THERMAL INACTIVATION OF PANTOTHENASE FROM *PSEUDOMONAS FLUORESCENS*

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Summary

Protection of pantothenase (pantothenate amidohydrolase, EC 3.5.1.22) against thermal inactivation by ligands was studied. The protective properties of 162 different small-molecule compounds were tested to find the most effective compounds. The best protective results were obtained with oxamate, 3-oxoglutarate, 2-oxomalonate, oxalate and oxaloacetate, in that order.

The protection constants (π) of certain metabolic intermediates were estimated using differential thermal inactivation. Generally they were observed to be lower than the corresponding inhibition constants. The ΔH of the oxalate binding derived from π values was —165 kJ/mol. Normally, the activation energy of thermal inactivation of pantothenase in the absence of protective compounds is 220 kJ/mol, and the protective ligands enhance the measured activation energies of inactivation.

Introduction

The ability of ligands to protect enzymes against thermal inactivation may be utilized to measure the protection constant (dissociation constant) of the ligand-enzyme complex [1,2]. Pantothenase (pantothenate amidohydrolase, EC 3.5.1.22) from *Pseudomonas fluorescens* is well protected against thermal inactivation in vitro by e.g. oxalate and in vivo in the presence of good carbon sources [3]. In the present investigation a large number of compounds was tested to find the most effective protectors of pantothenase, the protection constants were then measured and the obtained constants were compared with the inhibition constants of the same ligands.

Materials and Methods

Pantothenase preparation. Pantothenase was purified from *Ps. fluorescens* UK-1 as described elsewhere [4]. 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 column.

Reagents. The compounds employed in search of protecting or reactivating compounds were obtained from: Boehringer Mannheim GmbH, Mannheim, Germany; British Drug Houses Ltd., Poole, U.K.; Fluka A.G., Buchs, Switzerland; E. Merck A.G., Darmstadt, Germany; or Sigma Chemical Co., St. Louis, Mo., U.S.A. In all cases the purest commercial preparations available were used. 25 mM solutions of these compounds were prepared whereafter the pH was adjusted to 7 with 5 M KOH or 2.5 M H_2SO_4.

Pantothenase assay. The activity of pantothenase was determined as described previously [5]. The reaction mixture, 125 μl in volume, contained 25 μl of enzyme solution, 25 μl of buffer solution (usually 100 mM potassium phosphate pH 6.8), 25 μl of 150 mM potassium pantothenate, 25 μl of the solution of [1-^{14}C]pantothenate (80 000 dpm), and 25 μl of the solution of the added compound.

Inactivation and reactivation. The inactivation mixture, 75 μl in volume, contained 25 μl of enzyme solution (50—75 μg/ml, 6—9 nkat/ml), 25 μl of 100 mM potassium phosphate pH 6.8 and 25 μl of the solution of the added compound (usually 25 mM). The inactivation occurred in a water bath, and it was terminated by moving the test tubes into a water bath at 20°C and by pipetting 50 μl of ice-cold, 75 mM pantothenate containing the radioactive pantothenate (80 000 dpm).

Calculations. The Wang 2200 mini-computer was used in calculating the enzyme activities, protection constants, as well as the activation energies.

Results

Protective compounds

In order to test the existence of protective ligands, pantothenase was thermally inactivated in the presence of various compounds. The compounds tested, 162 in all, belonged to the following compound groups: carboxylic acids (29), alcohols, aldehydes and carbohydrates (17), amino acids (30), amines and other nitrogen compounds (16), coenzymes, nucleotides and related compounds (34), sulphydryl compounds (6), phosphate esters (12), pantothenate metabolites and analogs (8), and inorganic salts and miscellaneous compounds (10). Table I presents a list of the compounds with inhibitory or protective effects. The concentration of the compounds during inactivation was 8.3 mM which exceeds the concentration of most metabolites in bacterial cells [6—10]. Acetyl-CoA (the end product of β-alanine degradation) was also tested at 2 mM concentration, and it was found to possess a moderate protective effect (30—35% of the original). Most of this effect can, however, be ascribed to acetate entering the solution when preparing acetyl-CoA. In Table I the protectors, as a rule, serve as inhibitors, especially the most effective protective compounds are highly effective inhibitors. Apart from very few exceptions, the protective compounds proved to be carboxylic acids; the most effective protectors like 3-oxobutyrate, 2-oxomalonate and oxaloacetate having oxygen in α- or β-position.

TABLE I

INHIBITION AND PROTECTION AGAINST HEAT INACTIVATION

Pantothenase was inactivated for 30 min at 35°C in the presence of various compounds. 25 mM solutions of the compounds tested were prepared and adjusted to pH 6.8—7.0 with KOH or H_2SO_4. The concentration of the compounds was 8.3 mM during inactivation. The buffer was 100 mM potassium phosphate pH 6.8. The amount of pantothenase was about 17 μg/ml during the inactivation and 10 μg/ml during the activity measurements. The only compounds listed are those for which the inhibited activity level was below 80% of the uninhibited level or for which the remaining activity after inactivation was over 30%.

Compound	Inhibition (% activity of control)	Protection (% activity remaining)
None	100	8
Pantothenate (substrate)	(100)	14
Oxamate	10	80
2-Oxomalonate	17	78
Oxalate	21	74
Trifluoroacetate	25	37
3-Oxoglutarate	26	79
Oxaloacetate	33	72
Phosphoenolpyruvate	40	18
3-Hydroxybutyrate	45	10
2-Oxoglutarate	57	56
Pyruvate	61	44
Malate	63	48
2,2-Dimethylglutarate	63	10
Malonate	65	52
2-Oxoisovalerate	68	8
Glyoxylate	68	35
Tartrate	71	31
Thioglycollate	73	33
Acetate	74	25
Aldopantoate	77	17
Fumarate	79	40
Maleate	84	30
Methylmalonate	90	33
Diethanolamine	42	9
Ethylendiamine	56	17
o-Phenylendiamine	62	14
Histamine	100	55
Pantethine	70	25
ω-Methylpantothenate	77	41
Propanol	37	19
Phosphoethanolamine	49	8
Glutathion (GSH)	58	5
Acetoin	67	11
Glyceraldehyde	73	3
Nicotinic acid	75	34
Kynurenic acid	81	43
Kynurenine	100	34
Cl^- (10 mM)	79	16

Fig. 1 presents the time course of the thermal inactivation of pantothenase. As a rule, the inactivation follows a first-order course. Phosphate acts protectively as compared with Mops, and the most marked lability could be observed in unbuffered K_2SO_4.

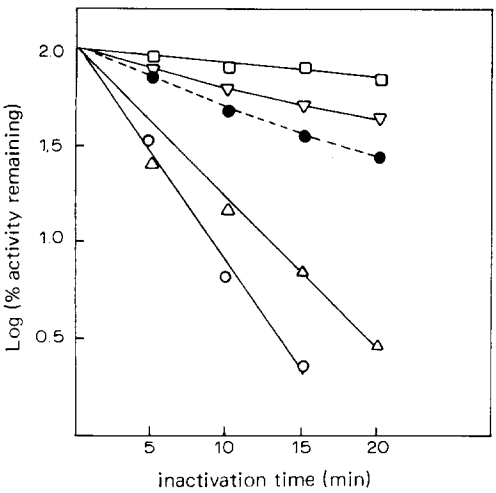

Fig. 1. Time course of thermal inactivation of pantothenase. The inactivation mixtures contained in a total volume of 75 μl the enzyme (17 μg/ml), the buffer, and the added compound. After inactivation at 34°C the test tubes were removed to 20°C and the enzyme assay was started at the same time by pipetting the substrate. The buffers and the added compounds were as follows: ●, potassium phosphate pH 7.05, 33 mM in the inactivation mixture, no added compound; ▽, the above phosphate buffer and 1.7 mM sodium pyruvate; □, the above phosphate buffer and 1.7 mM potassium oxalate; △, 17 mM morpholinopropane sulphonate (K^+ salt) pH 7.0, no additions; ○, only the enzyme in 5 mM K_2SO_4 in the inactivation mixture.

TABLE II

PROTECTION CONSTANTS FROM DIFFERENTIAL THERMAL INACTIVATION AND INHIBITION CONSTANTS FOR SOME LIGANDS

The experiment was carried out as described in Fig. 2. Inactivation stayed for 20 min at 35°C; the buffer was 100 mM potassium phosphate pH 7.05. The value of k_0 was 0.072 min^{-1}. The one-subunit theory was used in the calculation of the protection constants and consequently the derived π^{app} values may be lower than the real π values [12]. v.s., very small.

Compound	Protection constant (mM)	Inhibition constant (mM)	k_∞ (min^{-1})
Oxalate	1.15	1.67	
Oxalate	0.97	1.70	
Oxalate	0.74	1.77	v.s.
Oxalate, buffer 50 mM morpholino-propane sulphonate pH 6.90	0.44	1.11	
Oxaloacetate	1.21	2.16	v.s.
Pyruvate	2.6	6.0	0.017
Malate	2.4	10	0.011
Acetate	5.0	21	0.019
Fumarate	2.8	24	0.025
Succinate	2.1	93	0.050

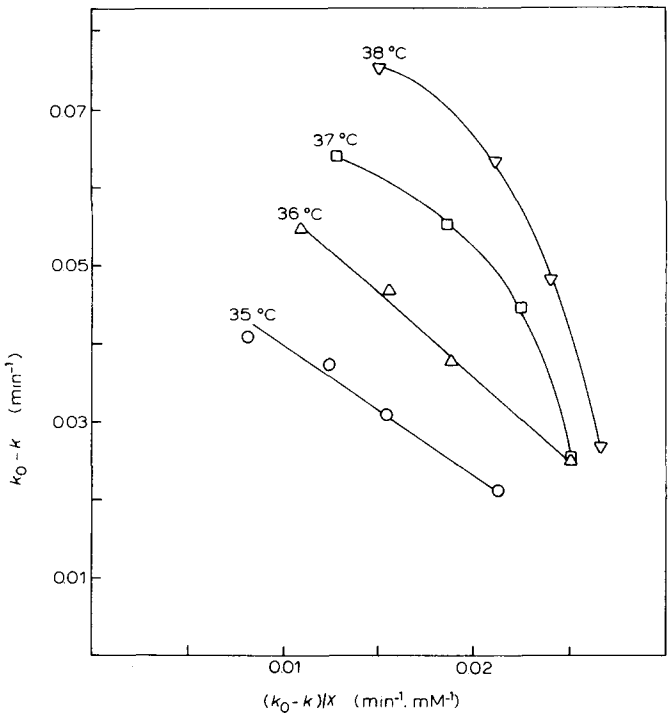

Fig. 2. Determination of the protection constants of oxalate by the differential inactivation technique. Pantothenase was inactivated for 20 min at temperatures, viz. 35°C (○), 36°C (△), 37°C (□), or 38°C (▽). The inactivation mixture contained 25 μl of the enzyme (180 μg/ml) in 5 mM potassium sulphate, 25 μl of the buffer (100 mM potassium phosphate pH 7.2), and 25 μl of potassium oxalate in concentrations 0, 1, 2, 3 or 5 mM. At the end of the inactivation the test tubes were removed to a water bath at 20°C and the enzyme assay was started. The values of π derived by linear regression are 1.53 mM at 35°C, 2.13 mM at 36°C, 2.95 mM at 37°C and 3.94 mM at 38°C.

Determination of protection constants

The method described by Chuang and Bell [11] for determining the protection constant, π, was used. Pantothenase was thermally inactivated in the presence of various concentrations of oxalate or other compounds. The rate constant (k) of the inactivation was calculated according to the equation $k = 1/t \cdot \ln 100/P$, where t is the inactivation time and P is the percent activity remaining. According to the theory of Chuang and Bell [11] the plotting of $k_0 - k$ vs. $(k_0 - k)/X$ should give a straight line whose slope is $-\pi$ (k_0 is the k without any ligand, and X is the ligand concentration). In Fig. 2 the protection constants of oxalate-caused protection have been determined at different temperatures. The plots were linear below 36°C but at 37°C or a higher temperature the plots curved. Table II presents the values of the protection constants of various compounds as well as of k_∞, the inactivation rate constant of the ligand-enzyme complex. Both the protection constants and the k_∞ values are in good correlation with the inhibition constants of the same compounds (when determined at 20°C). Fig. 3 shows the temperature dependence of π of oxalate-caused protection. π is rather significantly dependent on temperature, the calculated ΔH value of oxalate binding being −165 kJ/mol. The π values are lower at pH 6.5

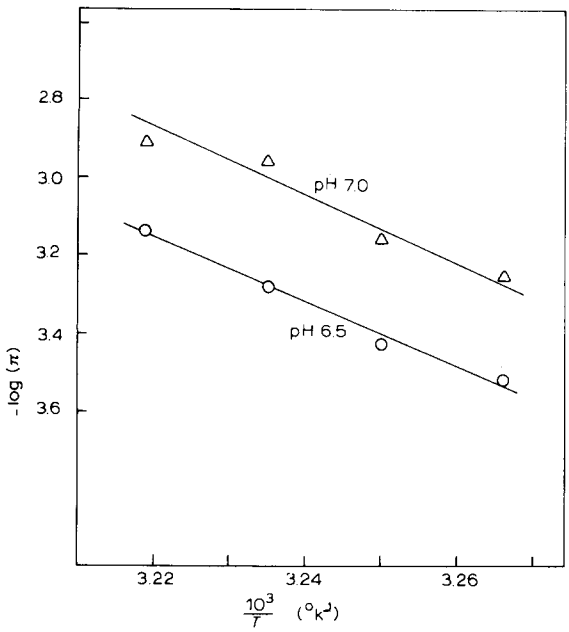

Fig. 3. Van 't Hoff plots relating π to inactivation temperature. The values of π were assayed as in Fig. 2. The inactivation temperatures varied from 33° to 37°C, and the buffers were 100 mM potassium phosphate pH 6.5 (○) or pH 7.0 (△). A ΔH value of −165 kJ/mol was calculated from the slopers of the lines.

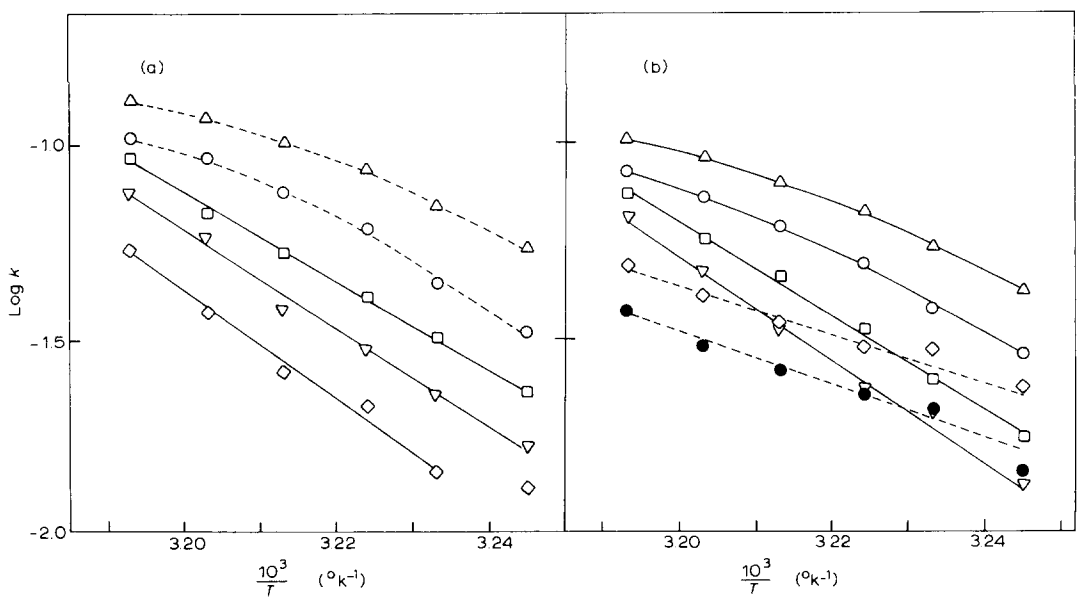

Fig. 4. Arrhenius plots of the inactivation of pantothenase in the presence of some metabolites. (a) Effect of oxalate concentration on inactivation. Pantothenase was inactivated for 20 min at various temperatures in test tubes containing 25 µl of pantothenase solution (180 µg/ml), 25 µl of 100 mM potassium phosphate pH 7.2 and 25 µl of solution of potassium oxalate. The oxalate concentrations in the inactivation mixture were 0 (△), 1 mM (○), 2 mM (□), 3 mM (▽) or 5 mM (◇). The remaining enzyme activity after inactivation was measured as usual. (b) Effect of some compounds on inactivation. The experiment was carried out as in (a) but instead of oxalate the following compounds were present in the inactivation mixture in 10 mM concentrations: sodium succinate (○), potassium acetate (◇), potassium fumarate (□), sodium pyruvate (●), potassium malate (▽) and no added compound (△).

than at pH 7.0, corresponding well to the dependence of the inhibition constants on pH [5].

Activation energies of thermal inactivation

Some measurements of the activation energies of the thermal inactivation of pantothenase are presented in Fig. 4. The Arrhenius plots tend to be curved when no ligands or very weak protectors are present, but with protective ligands the lines appear to be straight. Typical values of the activation energies of inactivation, calculated from the slopes of the Arrhenius plots, are about 220—240 kJ/mol in the absence of any protective effects of the ligands. Somewhat higher values (250—350 kJ/mol) are found in the presence of protective compounds or when pH is elevated to 8 or when the phosphate concentration is reduced to 5 mM. With 2 mM oxalate at pH 6.5 the activation energy rises as high as to 490 kJ/mol. The addition of bovine serum albumin has no apparent effect on the activation energy.

Discussion

One of the aims in investigation of the protecting compounds was to test whether the ligand-caused protection mechanism might account for the protection of pantothenase in whole cells in the presence of good carbon sources (or for the lack of protection with lacking carbon sources) [3]. Among the protecting compounds, oxaloacetate, pyruvate, malate and glyoxylate belong to the category of central metabolic intermediates; also the other central dicarboxylic acids show some protecting effects. None of these alone is sufficient to account for the effective protection because the concentrations in the bacterial cells are low [9,10], but if their effects are cumulative they could achieve an appreciable protection. Possible cumulative or even synergistic effects have so far remained obscure.

Both the protection constants of the various compounds and the k_∞ values are in good correlation with the inhibition constants of the same compounds (Table II). Protection and inhibition seem to result from the same ligand binding to the enzyme, which notion is well supported by the similar pH dependences of the π and K_i values.

As described in another study in this series [12], the protection constants should be calculated in a different way if the protein has several binding sites for the ligand (several subunits). In the protection of pantothenase by oxalate the protection constant determined assuming one-ligand binding is about half the correct π value [12]. Although the method of determining the π values used in the present paper gives too low values, it is practical to use this rather than "the exact method" for certain reasons. The measurement is much easier when the use of very low ligand concentrations is not necessary. The deviation of the results in "the exact method" is more marked. Much the same relative differences in π values are derived with this simple method. So if the various π values are to be compared with each other, the results obtained with the simple method are equally reliable. The real π value derived for oxalate-caused protection at 36°C, 2.4 mM, is still considerably lower than the inhibition constant at 36°C, 6.3 mM [12]. The cause of the π values being lower than the K_i

values remains obscure. The main differences in the measurement conditions of the constants are that the π values are measured over 35° C without substrate and the K_i values between 20 and 30° C in the presence of the substrate.

The inactivation of an enzyme in the presence of a protective ligand occurs in two separate reactions, both from free enzyme (rate constant k_0) and from enzyme-ligand complex (k_∞). If the activation energy of inactivation in the presence of a ligand is to be measured, the k_∞ should be measured at different temperatures. This is, however, very difficult on pantothenase because the estimation of k_∞ is done from $k_0 - k_\infty$ and the deviation then becomes big, and because the protectors of pantothenase are inhibitors. Due to these restrictions the activation energy values of pantothenase inactivation in the presence of protective compounds cannot be regarded as anything but indicative. The conclusions to be drawn from the measurements are that the protective ligands enhance the activation energy of inactivation of pantothenase and that the values can be elevated by ligands to the level detected in whole cells [3].

Furthermore, the π values being lower than the K_i values, also other anomalous features exist in the kinetics of the thermal inactivation of pantothenase. The strong curving of the plots in the π determination (Fig. 2) above 37° C is not in accordance with the theory. Also the curvature of the Arrhenius plots in the absence of ligands is abnormal, and further the ΔH of oxalate binding from Fig. 3, −165 kJ/mol, is essentially different from the corresponding ΔH from K_i values, −60 kJ/mol [12]. The detailed causes of these anomalies are unknown, but they are no doubt associated with the general character of the process of thermal inactivation. It is a multiphased event where even the rate-restricting stage can be changed with a change in temperature or in ligand binding, and the ligand can be bound except to the native form also to the intermediate stages.

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