Pantothenases from pseudomonads produce either pantoyl lactone or pantoic acid

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A pseudomonad was separated having a pantothenase that produced β -alanine and pantoic acid. The pantothenase from an old strain, *Pseudomonas fluorescens* UK-1, was shown to produce β -alanine and pantoyl lactone. The pantoic acid-producing pantothenase was characterized and compared with the pantothenase from the strain UK-1. The M_r was 240 000; it apparently consists of four subunits. The K_m value for pantothenate is 3 mM. The enzymic activity is affected by an ionizable group of pK 8.4, the enzyme is active at higher pH, and V but not K_m is affected by pH. This pantothenase is not inhibited by di-isopropyl phosphorofluoridate or phenylmethanesulphonyl fluoride, unlike the enzyme from the strain UK-1. Both pantothenases are inhibited by *m*-aminophenylboronic acid, oxalate, oxaloacetate and Cl^- ions. The pantoic acid-producing pantothenase is inhibited also by SO_4^{2-} ions. The strong inhibitions by many compounds make this pantothenase unsuitable for the assay of pantothenic acid.

INTRODUCTION

In the pantothenase (EC 3.5.1.22)-catalysed reaction pantothenic acid is decomposed to β -alanine and pantoic acid (Nurmikko *et al.*, 1966). The enzyme has been isolated from a couple of strains of *Pseudomonas fluorescens* (Puisto & Nurmikko, 1970; Airas *et al.*, 1976). In general, all organisms that grow on pantothenate as a sole carbon source must have pantothenase. In the classification system of Stanier *et al.* (1966) almost all the pseudomonads utilizing pantothenate fall into the biotype C of *Pseudomonas fluorescens*.

Originally, the product of the pantothenase-catalysed reaction was suggested to be pantoic acid (Nurmikko *et al.*, 1966). In the bacterial strain used in the pantothenase-based assay of pantothenate (Airas, 1983) the product proved to be D-pantoyl lactone. In a search for new bacterial strains for the assay of pantothenate, a strain was isolated whose pantothenase produced free pantoic acid. The product of the pantothenase-catalysed reaction can thus be either D-pantoyl lactone or free D-pantoic acid. In the present paper the pantothenases were isolated from two bacterial strains, and some properties of the enzymes were determined.

EXPERIMENTAL

Chemicals

Calcium pantothenate was from Merck (Darmstadt, Germany); Ca^{2+} ions were removed by an ion-exchanger (Dowex 50), and the pantothenic acid was titrated with KOH to pH 7. D-Pantoyl lactone [D(-)-2-hydroxy-3,3-dimethyl- γ -butyrolactone] was obtained from Fluka (Buchs, Switzerland); D-pantoic acid potassium salt was prepared from the lactone by keeping the pH of the solution between 10 and 11 by continuous additions of KOH during about 2 h at room temperature. Sodium [1- ^{14}C]pantothenate and β -[1- ^{14}C]alanine were from New England Nuclear (Dreieichenhain, Germany).

Enzyme preparations

The pantothenase from *Ps. fluorescens* UK-1 (N.C.I.B. 12017) was prepared as described previously (Airas,

1979). The bacterial strain *Pseudomonas* PS-21 was cultivated for the preparation of pantothenase as described for the strain UK-1. The cells were broken with a French press, the suspension was centrifuged for removal of the cell debris, and thereafter the chromatography on DEAE-Sephadex A-50 was accomplished as described previously (Airas, 1979). After the DEAE-Sephadex chromatography the pantothenase was precipitated by making the solution 2.5 M with respect to $(\text{NH}_4)_2\text{SO}_4$ (pH 7.4). The precipitate was collected by centrifugation and dissolved in 6 ml of water. A column (volume 35 ml) of Sepharose CL-6B was prepared in 2.6 M- $(\text{NH}_4)_2\text{SO}_4$, pH 7.4. The enzyme sample was applied to the column and mixed in about one-half of the volume of the gel. The column was eluted with a gradient from 2.6 M- to 1.0 M- $(\text{NH}_4)_2\text{SO}_4$, pH 7.4 (total volume 300 ml). The pantothenase-containing fractions [at 1.4 M- $(\text{NH}_4)_2\text{SO}_4$] were collected and precipitated by 3 M- $(\text{NH}_4)_2\text{SO}_4$.

A column (1.6 cm $\times$ 81 cm; 163 ml) of Ultrogel AcA 34 (LKB) was prepared in 10 mM- K_2SO_4 . The enzyme sample in 2.4 ml was applied to the column and the elution was accomplished with 10 mM- K_2SO_4 . The peak of pantothenase emerged at an elution volume of 88 ml.

Enzyme assays

The pantothenase activities were assayed as described previously (Airas, 1979). The reaction mixtures contained potassium D-pantothenate (30 mM, or as otherwise noted), D-[1- ^{14}C]pantothenate (5000 Bq/100 μl), buffer (50 mM-Tris/12.5 mM- H_2SO_4 , pH 8.1, for pantothenase UK-1 and 25 mM-Tris/25 mM-Tricine, pH 8.1, for pantothenase PS-21) and the enzyme. The assay temperature was 25 $^\circ\text{C}$. The fixed-time assay took 10 min. The enzyme concentration was chosen to give linear reaction progress curves for over 30 min. Tricine was used instead of H_2SO_4 to adjust the pH of the reaction mixtures of pantothenase PS-21 to avoid the sulphate inhibition of this enzyme (see the Results section). The reaction rates of the pantothenase UK-1 reaction were found to be unchanged whether the pH was adjusted with H_2SO_4 or Tricine. The radioactive β -alanine produced was

Table 1. Purification of pantothenase from *Pseudomonas* PS-21

For experimental details see the text. Enzyme activities were determined at pH 9 in 50 mM-diethanolamine/H₂SO₄ buffer.

	Volume (ml)	Protein (mg)	Enzyme activity (μ mol/min)	Specific activity (μ mol/min per mg of protein)
Cell extract	32	633	165	0.26
After DEAE-Sephadex chromatography	112	147	130	0.88
After Sepharose column chromatography	2.3	9.4	74	7.9
After Ultrogel AcA 34 chromatography	24.6	3.9	53.3	13.7

separated from pantothenate by paper chromatography in butan-1-ol/acetic acid/water (25:4:10, by vol.).

The rate of the reverse reaction was assayed similarly to the forward reaction but the reaction mixtures contained D-pantoyl lactone (or potassium D-pantoate) (varied between 10 and 50 mM), β -[1-¹⁴C]alanine (5000 Bq/100 μ l), buffer as above, β -alanine (varied between 1 and 10 mM) and the enzyme. The radioactivity of the pantothenate spot from the paper chromatogram was counted.

The rate of the pantothenate/ β -[1-¹⁴C]alanine exchange reaction was measured as in the normal forward reaction above but the reaction mixture contained radioactive β -alanine instead of the radioactive pantothenate, and the radioactivity transferred to pantothenate was measured.

In the determination of the equilibrium constant the reaction mixture contained the buffer, β -[¹⁴C]alanine and various concentrations of pantothenate, β -alanine and pantoyl lactone, e.g. 1.7 mM-pantothenate, 10 mM- β -alanine and 10 mM-pantoyl lactone. For pantothenase PS-21 potassium pantoate was substituted for pantoyl lactone, and no pantothenate was added. The reaction mixtures were incubated overnight at 25 °C, and the radioactivities of both pantothenate and β -alanine spots were counted. The equilibrium constants were calculated according to eqn. (1):

$$K_{eq.} = \frac{b \cdot p}{s} = \frac{(p_0 + \alpha p_0 + s_0 - \alpha b_0)}{\alpha(1 + \alpha)} \quad (1)$$

where s , b and p are the concentrations (at equilibrium) and s_0 , b_0 and p_0 are the original concentrations of pantothenate, β -alanine and pantoyl lactone respectively, and α is the measured s/b .

The enzyme inhibitions by phenylmethanesulphonyl fluoride and di-isopropyl phosphorofluoridate were measured by incubating the enzyme with 100 μ M inhibitor and 20 mM-potassium phosphate, pH 7.4, at 20 °C, and normal enzyme assays of the incubation mixture were done at various intervals up to 60 min.

General methods

Polyacrylamide-gel electrophoresis (in non-denaturing conditions) was performed in 7.5% gels as described by Davis (1964). SDS/polyacrylamide-gel electrophoresis was carried out in 7.5% gels as described by Weber *et al.* (1972).

The samples for determination of the n.m.r. spectra contained the reaction mixtures of the buffer, 30 mM-potassium pantothenate and the enzyme, and the hydrolysis of the substrate was run to the end for 24 h at

room temperature. The buffers were 20 mM-potassium phosphate, pH 7.3, for pantothenase PS-21 (pH did not change) and 50 mM-potassium phosphate, pH 6.7, for pantothenase UK-1 (pH was 7.66 at the end of the reaction). The standards β -alanine, D-pantoyl lactone and potassium D-pantoate were adjusted to pH 7.3. ²H₂O (10%, v/v) was added to all of the samples. The ¹³C-n.m.r. spectra were collected on a Jeol GX-400 spectrometer operating at 100 MHz, and the chemical shifts are given in p.p.m. from external tetramethylsilane.

RESULTS

Bacteria utilizing pantothenic acid

Bacteria growing with pantothenate as the sole source of carbon were isolated from various sea, river or ditch waters. Portions (100 ml) of the water were each filtered on a Millipore HAWP bacterial filter, then the filters were placed on pantothenate/agar plates, and the plates were incubated at 25 °C. Several hundreds of colonies could be found on the plates from any water source. A greater majority of the colonies were of small, motile, fluorescent-colour-producing bacteria (not classified, but most probably *Pseudomonas fluorescens* and *Pseudomonas putida*). Twenty colonies were selected on the basis of different colour or morphology of the colonies. Two of these were very slowly growing moulds. One bacterial strain was microscopically clearly different from pseudomonads, and 17 strains were morphologically similar to the pseudomonads although all of these appeared to differ from each other. The pantothenase activities and the K_m values were measured from the bacterial strains. Finally one strain was selected whose pantothenase had a low K_m value. Attempted classification according to the Oxi-Ferm tube system (Roche) gave not a single strain but a group of pseudomonads. The strain has not been further characterized. Therefore a strain number, PS-21, is used.

Purification of pantothenase from *Pseudomonas* PS-21

The purification of the pantothenase from *Pseudomonas* PS-21 was accomplished by using chromatography on DEAE-Sephadex, solubilization of the (NH₄)₂SO₄-fraction precipitate in a Sepharose column and gel filtration in an Ultrogel AcA 34 column. The details of the purification are described in the Experimental section, and the purification is summarized in Table 1. The enzyme preparation was about 70% pure as estimated from polyacrylamide-gel electrophoresis (without SDS). The gels after electrophoresis both with and

Scheme 1. Reactions catalysed by pantothenases UK-1 and PS-21

without SDS showed one major band and several minor impurities.

The M_r of pantothenase PS-21 was assayed by gel filtration on Ultrogel AcA 34 to be 240000. A subunit M_r of 58 500 was obtained from the SDS/polyacrylamide-gel electrophoresis, which suggests four subunits of equal size. The M_r of the pantothenase UK-1 is 100000, and this enzyme consists of two equal subunits (Airas *et al.*, 1976).

Products of the pantothenase reactions

Three types of experimental evidence show that the products of the pantothenase-catalysed reactions are different in the strains UK-1 and PS-21. One of the products, β -alanine, is the same, but pantothenase UK-1 produces D-pantoyl lactone and pantothenase PS-21 produces D-pantoic acid (Scheme 1).

(a) The pH is not changed in the reaction catalysed by pantothenase PS-21. Equal amounts of base (the amino group of β -alanine) and acid (the carboxy group of pantoic acid) are produced. The pH is not changed either when D-pantoyl lactone is used as the substrate in the reverse reaction, showing that the lactone is not a dissociable intermediate in the pantothenase PS-21-catalysed reaction, and that pantothenase PS-21 does not contain two separate enzymes pantothenase and lactonase.

In the reaction catalysed by pantothenase UK-1 the pH rises; base but no acid is produced. When the reaction mixture was continuously titrated to keep the pH constant, the molar consumption of acid was equal to the production of β -alanine, measured by use of the radioactive substrate.

(b) D-Pantoyl lactone reacts in the reverse reaction catalysed by the pantothenase UK-1, pantothenic acid being formed in the presence of β -alanine and the lactone. On the other hand, no reverse reaction with D-pantoic acid could be measured (i.e. the reaction with the pantate was less than 2% of that with the lactone).

Pantothenase PS-21 shows no reverse reaction with D-pantoyl lactone. A slow reverse reaction with pantoic acid can be measured, but the reaction soon reaches an equilibrium (Table 2).

(c) The ^{13}C -n.m.r. spectra were obtained for the reaction mixtures of both enzymes when the reactions had been run to the end. The n.m.r. spectrum of the reaction mixture of the UK-1 enzyme was a sum of the chemical shifts of β -alanine (33.8, 37.0 and 178.5 p.p.m. from tetramethylsilane) and D-pantoyl lactone (18.3, 21.5, 40.7, 75.8 and 77.1 p.p.m.). Correspondingly, the spectrum of the reaction mixture of the PS-21 enzyme showed the chemical shifts of β -alanine and D-pantoate (19.8, 20.6, 36.9, 38.3, 69.1, 77.6 and 179.8 p.p.m.).

Table 2. Some kinetic constants for the pantothenases

	Pantothenase UK-1	Pantothenase PS-21
K_m for pantothenate	14.6 mM	3.2 mM
$k_{cat.}^*$	28.2 s ⁻¹	30 s ⁻¹ (pH 8.1) 55.6 s ⁻¹ (pH 9) (about 0.03 s ⁻¹)
$k_{cat.}$ (reverse reaction) [†]	3.0 s ⁻¹	
$v_{forward}/v_{exchange}^\ddagger$	2.4	12.0
Equilibrium constant	77×10^{-3} M	42 M

* $k_{cat.} = V/e$; the enzyme concentrations (e) were calculated by using the M_r values from Table 2. The buffer at pH 9 was 50 mM-diethanolamine/ H_2SO_4 .

[†] Calculated from V (pantoyl lactone concentration varied) of the reverse reaction at 10 mM- β -alanine.

[‡] At 2 mM-pantothenate, 2 mM- β -alanine and pH 8.1.

Fig. 1. Determination of the K_m of pantothenase PS-21 at different concentrations of β -alanine

Enzymic properties of pantothenase from *Pseudomonas* PS-21

The bacterial strain *Pseudomonas* PS-21 was chosen because of the low K_m value of its pantothenase. Fig. 1 shows the K_m determination at different concentrations of β -alanine. The K_m value for pantothenate is about 3 mM in the absence of β -alanine and much higher in the presence of β -alanine.

Fig. 2 shows the rate of the pantothenate hydrolysis as a function of pH. pH clearly affects the rate of hydrolysis, the optimum pH being 9.0–9.2. The pK value of an

Fig. 2. Effect of pH on the reaction velocity of pantothenase PS-21

The buffers contained 25 mM-Mops, -Tricine, -Taps and -piperazine, and the pH values were adjusted with KOH. In (b) the pH-dependence:

$$v = \frac{V \left(1 + \frac{[H^+]}{K_a} \right)}{1 + \frac{K_m}{s}}$$

is written in the form:

$$\frac{1}{v[H^+]} = \frac{1 + \frac{K_m}{s}}{V} \cdot \frac{1}{[H^+]} + \frac{1 + \frac{K_m}{s}}{V} \cdot \frac{1}{K_a}$$

ionizable group affecting the rate between pH 7.5 and 9 is 8.4 (Fig. 2b), the V but not K_m is affected, and the enzyme is active on the basic side of this pK value. An increase in the ionic strength lowers the pK value. pK values of 7.8 and 7.65 were obtained when assays similar to those shown in Fig. 2 were done in the presence of 50 mM- and 200 mM-potassium phosphate buffer respectively. (Buffers similar to those used in the experiments of Fig. 2 were used; the pH values of the phosphate solutions were adjusted to give the desired pH in the reaction mixture.) Preincubation of the enzyme with the buffers did not cause inactivation, even at the highest pH values 9.5 and 10.

Pantothenase UK-1 reacts with the serine-proteinase inhibitors phenylmethanesulphonyl fluoride and di-isopropyl phosphorofluoridate, giving rate constants $420 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $460 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively (at pH 7.4 and at 20°C). The same inhibitor under the same conditions did not inactivate pantothenase PS-21 at all.

The two pantothenases are inhibited similarly by *m*-aminobenzenboronic acid, oxalate or oxaloacetate (Table 3). KCl also inhibits both enzymes, 50% inhibition being attained at 50 mM-KCl for pantothenase UK-1 and at 80 mM-KCl for pantothenase PS-21. K_2SO_4 inhibits pantothenase PS-21 strongly at low substrate concentrations (50% inhibition at 15 mM- K_2SO_4 at 2 mM substrate) but less strongly at high substrate concentrations. This inhibition did not follow the normal hyperbolic inhibition kinetics, and so a K_i value cannot be expressed. Neither enzyme is inhibited by EDTA or potassium phosphate. 2-Mercaptoethanol or dithiothreitol did not affect the enzyme activities. 4-Chloromercuribenzoic acid ($5 \mu\text{M}$) caused a small increase in the K_m value but no change in V . Apparently no thiol group exists in a crucial position for the enzymic activity. Table 3 summarizes the properties of the two pantothenases.

DISCUSSION

Properties common to pantothenase UK-1 and the serine proteinases include similar pH-activity relationships and inhibitions with di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride. Pantothenase PS-21 differs in all of these properties. Therefore the active sites in the two enzymes must differ from each

Table 3. Some molecular and enzymic properties of the pantothenases

	Pantothenase UK-1	Pantothenase PS-21
Product (in addition to β -alanine)	Pantoyl lactone	Pantoic acid
M_r	100 000	240 000
No. of subunits	2	4
pK value affecting the rate	6.8	8.4
Inhibition by:		
Phenylmethanesulphonyl fluoride	$k = 420 \text{ M}^{-1} \cdot \text{s}^{-1}$	None
Di-isopropyl phosphorofluoridate	$k = 460 \text{ M}^{-1} \cdot \text{s}^{-1}$	None
<i>m</i> -Aminobenzenboronic acid (competitive)	$K_i = 0.1 \text{ mM}$	$K_i = 0.14 \text{ mM}$
Oxalate (non-competitive)	$K_i = 1.8 \text{ mM}$	$K_i = 0.55 \text{ mM}$
Oxaloacetate (non-competitive)	$K_i = 2.2 \text{ mM}$	$K_i = 1.2 \text{ mM}$
KCl	50% at 50 mM	50% at 80 mM
K_2SO_4	None	50% at 15 mM
EDTA	None	None

Scheme 2. Deacylation of acyl-pantothenase UK-1

other, and pantothenase PS-21 differs from the serine proteinases. Most of the metalloproteinases have neutral or basic pH optima (Matsubara & Feder, 1971). Although pantothenase PS-21 has a basic pH optimum, it seems not to be similar to the metalloproteinases because of its total insensitivity to EDTA. The similarities between the two pantothenases include the inhibitions with *m*-aminobenzenboronic acid, oxalate, oxaloacetate and Cl^- ions. In addition, the existence and kinetics of the pantothenate/ β -[^{14}C]alanine exchange reaction suggests an acyl-enzyme mechanism for both pantothenases.

In earlier reports about pantothenase UK-1 (Airas, 1978, 1979, and references cited therein) no attention was paid to the form of the product, and the reaction was written to produce pantoic acid instead of the real product pantoyl lactone. Similarly the first report about the pantothenase from *Pseudomonas fluorescens* P-2 (Nurmikko *et al.*, 1966) did not examine the product. Since this bacterial strain is no longer available, the nature of the product cannot be determined. The pH-activity relationship of this enzyme is similar to that of pantothenase UK-1, but this does not establish that the product of the pantothenase P-2 reaction was pantoyl lactone.

The total reaction of pantothenase UK-1 does not necessarily include participation of water. In the serine proteinases a water molecule is functioning as a nucleophile in the deacylation (Kraut, 1977). The deacylation of the acyl-pantothenase into pantoyl lactone and free enzyme can be written without water, in which case the γ -hydroxy group of the pantoyl moiety would function as the nucleophile (Scheme 2). Unlike the situation with the UK-1 enzyme, a water molecule must be used in the deacylation of acyl-pantothenase PS-21. On the basis of the complete reactions catalysed by the two pantothenases, pantothenase PS-21 can be classified as a hydrolase and pantothenase UK-1 as a lyase.

The relatively high rate of the reverse reaction catalysed by pantothenase UK-1 (Table 2) raises the question whether this enzyme could function also in pantothenate synthesis when no pantothenase is present in the growth medium. If, quite arbitrarily, the pantoyl lactone con-

centration is 1 mM and the β -alanine concentration 1 mM, then the pantothenate concentration at the equilibrium would be 13 μM , calculated from the equilibrium constant in Table 2. Since these concentration values are reasonable for the intracellular concentrations, a role of the pantothenase in pantothenate synthesis is not excluded. An enzyme reducing oxopantoyl lactone to pantoyl lactone exists in some bacteria and yeast (Brown & Williamson, 1982), but its existence has not been reported in pseudomonads.

The goal of the present work was to find a pantothenase better suited to the pantothenate assay than pantothenase UK-1. The deficiencies of the latter are its relatively high K_m value and sensitivity to inhibition by various compounds (Airas, 1983). The advantage of pantothenase PS-21 is the low K_m value. However, the low rate of the pantothenate/ β -[^{14}C]alanine exchange reaction (Table 2) and inhibition by several compounds, e.g. SO_4^{2-} ions, make this enzyme a poor candidate for the pantothenate assay.

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