Kinetic Studies on Pantothenase from *Pseudomonas fluorescens*

EFFECTS OF pH ON SUBSTRATE AND INHIBITOR BINDING

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The velocity of the pantothenase-catalysed hydrolysis of pantothenate was studied over pH 5.5–9, and in the presence of oxalate or oxaloacetate as an inhibitor. The pH-dependence of the reaction can be described by a kinetic equation containing two ionizations of the enzyme, with one ionizable group located at the substrate-binding site, and the other at the inhibitor-binding site. The K_m value of pantothenase to pantothenate depends on the buffer used, and phosphate tends to give somewhat lower values than other buffers. K_m also depends on pH, the best activities being observed at basic pH values. The pH-independent K_m is 7.6 mM in phosphate buffer at 20°C; the corresponding K_m^{app} value at pH 7 is 15 mM. The pK value of the ionizable group at the substrate-binding site was measured by two methods: from the pH–rate profile and from the pH– K_m profile. pK is 7.0 in phosphate buffer at 20°C, ranging in various buffers between 6.9 and 7.3. The van't Hoff enthalpies of substrate binding and H^+ ion binding were –14 kJ/mol and –50 kJ/mol respectively. The inhibition by oxalate or oxaloacetate is of non-competitive type and depends on pH, the inhibitors being effective at acidic pH values. The pK value of the ionizable group at the inhibitor-binding site was derived from the measurements of the K_i values over the pH range 6–7.5. The pK value was 6.4 in oxaloacetate inhibition, the pH-independent K_i being 0.36 mM, and the corresponding K_m^{app} about 1.8 mM at pH 7. Phenylmethanesulphonyl fluoride was capable of inactivating pantothenase.

Pantothenase from *Pseudomonas fluorescens* (pantothenate amidohydrolase, EC 3.5.1.22) is an enzyme decomposing pantothenate into pantoate and β -alanine. The enzyme was first reported by Nurmikko *et al.* (1966). It is inducible by pantothenate and repressible by several metabolites (Mäntsälä & Nurmikko, 1970). Reports of the kinetic constants of pantothenase from *Ps. fluorescens* P-2 have been published on $K_m = 5$ mM at pH 7.5 at 28°C (Nurmikko *et al.*, 1966), and on the K_i values for such inhibitors as glyoxylate, oxaloacetate and pyruvate (Mäntsälä, 1970).

The purpose of the present paper is to investigate the kinetics of the pantothenase from *Ps. fluorescens* UK-1 with particular attention to the dependence of K_m and K_i values on pH and temperature.

Materials and Methods

Pantothenase preparation

The pantothenase was purified from *Ps. fluorescens* UK-1 by the method described in the preceding paper (Airas *et al.*, 1976).

Reagents

Calcium pantothenate was obtained from Fluka AG, Buchs, Switzerland. For substrate solutions

the Ca^{2+} was removed from pantothenate with an ion-exchanger (Dowex 50, H^+ form), pantothenic acid was then titrated with KOH to pH 7, and simultaneously, the exact concentration was fixed. The radioactive pantothenate came from New England Nuclear Corp., Dreieichenhain, Germany, it was purified with Dowex 50 in order to minimize the background caused by free β -alanine. Mops [3-(*N*-morpholino)propanesulphonic acid] was obtained from Serva, Heidelberg, Germany. Phenylmethanesulphonyl fluoride came from Sigma Chemical Co., St. Louis, MO, U.S.A. The standard buffer, Beckman pH 7.0 $\pm$ 0.02 (at 25°C), was purchased from Beckman Instruments Inc., Fullerton, CA, U.S.A. The pH of the buffers was adjusted at 100 mM concentration and the final buffer concentration in enzyme activity measurements was 20 mM.

Assay of pantothenase

The pantothenase activity was determined as described by Airas (1972) with slight modifications. The reaction mixture (125 μ l) contained the following components, 25 μ l of each (if not stated otherwise): 150 mM (30 mM in reaction mixture)-potassium pantothenate; solution of [$1-^{14}C$]pantothenate (80000 d.p.m./25 μ l); 100 mM (20 mM)-potassium phosphate, pH 7.0; solution of inhibitor (or water); and

the sample of pantothenase. The reaction occurred in small test tubes in a water bath at 20°C, and the duration of the reaction was normally 30 min. The reaction was stopped by pipetting a 25 μ l sample of the reaction mixture on to Whatman no. 1 chromatography paper located 1 cm from a surface of 30% (v/v) formic acid. The chromatograms were developed in butan-2-one/methanol/water/36% HCl (60:15:15:1, by vol.). R_F values: β -alanine, 0.30; pantothenate, 0.85. The duration of the development was 45 min. Also the previously (Airas, 1972) described solution butan-1-ol/acetic acid/water (25:4:10, by vol.) was utilized when developing the samples of particularly extensive experimental series. The β -alanine spots were cut off, dipped into scintillation liquid (Airas, 1972), and counted for radioactivity with a liquid-scintillation counter (Wallac Decem-NTL³¹⁴).

Calculation of enzyme activity

A Wang 2200 mini computer was used to calculate the enzyme activities from the radioactivity. The decrease of substrate concentration (s_0) during the reaction was taken into consideration in computations of v_0 from the integrated Michaelis equation by setting V equal in the normal integrated Michaelis equation $V_t = y + K_m \ln(s_0/s_0 - y)$ and the equation describing the initial situation $v_0 = V/(1 + K_m/s_0)$ (y = the decrease of substrate concentration in time t). The equation derived is:

$$v_0 = \frac{y + K_m \ln[s_0/(s_0 - y)]}{1 + (K_m/s_0)} \cdot \frac{1}{t} \quad (1)$$

Calculation of K_m

The K_m values were obtained either by the integrated Michaelis equation or by applying the direct linear-plot method described by Eisenthal & Cornish-Bowden (1974) to a computer program. In the latter method the K_m values are initially calculated for each possible pair of measurements (s_1, v_1 and s_2, v_2), and the median value of these K_m values represents the final K_m .

pK values from the curve v versus pH

When K_m^{app} is pH-dependent, and the enzyme is active on the basic side of the pK value, the reaction velocity of the enzyme is described by the following equation (Dixon & Webb, 1964):

$$v = \frac{V}{1 + \frac{K_m(1 + H/K_1)}{s}} \quad (2)$$

in which K_1 is the proton-dissociation constant, H is the H^+ ion concentration, and K_m is the real,

pH-independent Michaelis constant. [Later K_m^{app} will be the pH-dependent constant given by the equation $K_m^{app} = K_m(1 + H/K_1)$.] If at two pH values pH_1 (H_1, V_1 and v_1), and pH_2 (H_2, V_2 and v_2), V_1 is set equal to V_2 in eqn. (2), then eqn. (3) is derived:

$$K_{1(i,j)} = \frac{H_j v_j - H_i v_i}{(v_i - v_j)(1 + s/K_m)} \quad (3)$$

When the value of K_m is known, $K_{1(i,j)}$ can be defined for each possible pair of measurement points (H_1, v_1 and H_2, v_2). Then the median value of these $K_{1(i,j)}$ values is chosen to represent the final K_1 .

Phenylmethanesulphonyl fluoride inhibition

In the inhibition experiments the enzyme was incubated with 1 mM-phenylmethanesulphonyl fluoride at 10°C for various periods of time up to 4 h, then the enzyme activity was determined. During the reaction the test tubes contained 25 μ l of enzyme, 25 μ l of 100 mM-potassium phosphate, pH 6.95, and 25 μ l of 3 mM-phenylmethanesulphonyl fluoride in 10% (v/v) propan-2-ol. The enzyme reaction was commenced by pipetting the substrate as usual.

Results

K_m determinations

Table 1 represents some pantothenase K_m values in various buffers. A distinct difference prevails between the K_m values in phosphate buffer, and in Tris buffer. With increasing phosphate concentration the K_m value appears to decrease. This would suggest a responsibility of phosphate ion (HPO_4^{2-} or $H_2PO_4^-$) for the changes in K_m . When bovine serum albumin is added, the enzyme activity of the enzyme preparation used increases to some extent. However, no difference between the K_m values could be demonstrated when the K_m value was measured in the presence, and then in the absence of bovine serum albumin.

pH -dependence of K_m

Fig. 1 presents the enzyme activity against pH. A steep fall in activity is observed below pH 7. The substrate, pantothenate, has a pK value of about 4.2, but there is no pK near 7. Such a fall in activity may be due to the changes of K_m or V against pH (Dixon & Webb, 1964). Fig. 2 shows that in this case the fall can be ascribed to the changing K_m^{app} value. K_m^{app} is here determined at various pH values, plotted according to the integrated Michaelis equation, and then drawn as a secondary plot K_m^{app} versus H . The maximum velocity, V , from Fig. 2(a) is about equal (3.2 nmol/s) at different pH values. The values of K_m and pK_1 (in Mops buffer, from

Fig. 2) were 16.0mm and 7.12 respectively. In similar determinations the values of K_m were, in 20mm-potassium phosphate buffers, 7.6, 8.0 and 10.0mm, and the values of pK_1 were 6.78, 6.80 and 6.82. The pK_1 value was thus about 0.3 pH unit lower in phosphate buffer than in Mops buffer. The K_m value was markedly lower in phosphate buffer than in Mops buffer, corresponding well to the above difference in K_m^{app} values in phosphate buffer and in Tris buffer. Fig. 3 shows a collection of K_m^{app} values obtained from various determinations in phosphate buffer. By using the linear-regression method K_m was calculated as 7.8 ± 3.0 mm, and pK_1 as 6.84. A number of pK values of the ionizable group in the active centre were also calculated from the plot v versus pH as described in the Materials and Methods section, and the values are presented in Table 2. As a rule the pK values ranged between 7.0–7.3. The pK values in phosphate buffer were in

no way different from those determined in other buffers.

Inhibitors of pantothenase and the type of inhibition

Glyoxylate, oxaloacetate and pyruvate were observed by Mäntsälä (1970) to inhibit the pantothenase of *Ps. fluorescens* P-2. The inhibitors of the pantothenase from strain UK-1 were sought when measuring the protective ability of various compounds against thermal inactivation (R. K. Airas,

Table 1. K_m values of pantothenase to pantothenate

The concentration of potassium pantothenate was varied from 3.3mm to 40mm, the assay temperature was 20°C and the reaction was followed by taking samples from the reaction mixture at 5 min intervals.

Buffer	pH	K_m (mm)
10mm-Tris/ H_2SO_4	7.5	24
10mm-Tris/10mm-citric acid (KOH)	7.5	22
10mm-Sodium pyrophosphate (H_2SO_4)	7.5	18
5mm-Potassium phosphate	7.4	15
20mm-Potassium phosphate	7.4	13
60mm-Potassium phosphate	7.4	12.5

Fig. 1. pH-dependence of pantothenase activity

The reaction mixture contained 37.5 mm-substrate, and buffers as follows: $\square$, 20mm-potassium phosphate; $\circ$, 10mm-histidine hydrochloride/10mm-Tris, the pH of the buffer solution was first adjusted to 9.0 with KOH and then to the given pH values with HCl; $\bullet$, 10mm-tetrasodium pyrophosphate, pH adjusted with HCl.

Fig. 2. Determination of K_m^{app} values of pantothenase at different pH values

(a) K_m determinations at different pH values plotted according to the integrated Michaelis equation:

$$\frac{1}{t} \ln \left(\frac{s_0}{s_0 - y} \right) = \frac{V}{K_m} - \frac{1}{K_m} \cdot \frac{t}{y}$$

in which y is the decrease in substrate concentration during incubation. The buffer was 10mm-Mops (K^+ salt). The pH values were as indicated in the Figure. (b) Secondary plot of the K_m^{app} values against the H^+ ion concentration. The K_m^{app} values were calculated by a program of linear regression as well as the values of $K_m = 16.0$ and $pK = 7.12$ derived from the secondary plot.

Fig. 3. pH-dependence of K_m^{app} values of pantothenase in phosphate buffer

The values have been gathered from independent K_m determinations with different pantothenase preparations. Phosphate concentrations: $\circ$, 20mM; $\bullet$, 30mM; $\blacktriangle$, 40mM.

Table 2. pK values of pantothenase calculated from the dependence of the reaction velocity on pH

Enzyme activities were measured at different pH values and the pK values were calculated as described in the text. The assay temperature was 20°C when not indicated.

Buffer	Temperature (°C)	pK
Effect of temperature:		
60mM-Potassium phosphate	10	7.45
	15	7.25
	20	7.08
	25	7.00
20mM-Potassium phosphate	9	7.46
	12	7.41
	15	7.22
	18.3	7.05
	21	6.95
	24	6.89
Different buffers:		
10mM-Histidine/10mM-Tris (HCl)		7.15
10mM- α -Picoline/10mM-Tris (HCl)		7.26
10mM- α -Picoline/10mM-triethanolamine (HCl)		7.30
10mM-Citric acid/10mM-Tris (KOH)		7.18
10mM-Sodium pyrophosphate (HCl)		6.95
5mM-Glutamate/5mM-citrate/5mM-Tris (KOH)		7.09

unpublished work). Among the inhibitors, which as a rule belonged to carboxylic acids, oxamate, 2-oxomalonate and oxalate, 3-oxoglutarate and oxaloacetate were the most effective.

Fig. 4 presents the determination of the type of inhibition for oxalate. The inhibition was non-competitive. Also Mäntsälä (1970) reported that the inhibition was of non-competitive type for the pantothenase from strain P-2.

Fig. 4. Dixon plots of the inhibition of pantothenase by oxalate

Pantothenase activities in the presence of various concentrations of potassium oxalate were assayed at following substrate concentrations: 3.3 mM ($\bullet$), 5 mM (∇), 10mM (Δ), 20mM ($\square$), and 30mM ($\circ$). The buffer was 20mM-potassium phosphate, pH6.6.

pH-dependence of inhibition

The inhibition caused by oxalate or oxaloacetate is strongly dependent on pH. Fig. 5(a) presents the Dixon plots ($1/v$ versus i) representing the oxaloacetate inhibition at various pH values. These give the K_i^{app} values for which a secondary plot K_i^{app} versus $1/H$ is constructed. The pK value of the ionizable group at the binding site of the inhibitor is thus 6.4, and the pH-independent K_i is 0.36mM. Oxaloacetate has pK values of 2.3 and 4.4, so the pK derived is not due to the inhibitor itself. Table 3 gives certain pK values and K_i values derived from corresponding determinations. The values do not, as a rule, differ markedly from those above.

Kinetic equation

On the basis of the pH-dependences of K_m and K_i , the following complex equation was formed to describe the behaviour of pantothenase (E).

Fig. 5. pH-dependence of inhibition constants of oxaloacetate inhibition

(a) Dixon plots at different pH values. The buffer was 20mM-potassium phosphate at pH: 6.24 (○), 6.48 (●), 6.76 (Δ), 6.97 (□), 7.23 (▲), and 7.47 (▽). (b) Secondary plot of the K_i^{app} values against $1/H$.

Table 3. pH-independent K_i values and pK values of the ionizable group affecting the K_i value

The experiment was carried out as in Fig. 5.

Buffer	Inhibitor	pK	K_i (mM)
20mM-Potassium phosphate	Oxalate	6.35	0.31
20mM-Potassium phosphate	Oxaloacetate	6.39	0.36
10mM-Mops (K $^+$ salt)	Oxalate	6.10	0.20
10mM-Mops (K $^+$ salt)	Oxaloacetate	6.38	0.38

K_m and K_i are the pH-independent constants (K_m^{app} and K_i^{app} are pH-dependent). H and $\bar{H}$ represent the protons attached to substrate-binding site or inhibitor-binding site respectively. When forming this equation it was assumed that inhibitor (I) is attached only to the ionic form with a bound $\bar{H}$, and substrate (S) is attached only to the form without H . Further, let us assume that (1) the Michaelis conditions prevail; (2) that H or S bindings have no effect on the $\bar{H}$ or I binding and vice versa; (3) that the binding of $\bar{H}$ has no effect on the reaction velocity (i.e. $v = kES + kHES$); and (4) that $\bar{H}EIS$ is not decomposed into the products (P).

Fig. 6. Variation of K_m^{app} with temperature

K_m^{app} determinations were carried out at different temperatures, the results were plotted according to the integrated Michaelis equation, and K_m^{app} values were calculated by linear regression. The buffer was 20mM-potassium phosphate, pH6.95. The ΔH value of substrate binding estimated from the slope of the line is 15 kJ/mol.

Normal treatment methods of enzyme kinetics then give the rate equation:

$$v = V / \left[\left(1 + \frac{K_m(1 + H/K_1)}{s} \right) \left(1 + \frac{i}{K_i(1 + K_2/H)} \right) \right] \quad (5)$$

At a constant pH the equation may be written as in the normal equation of non-competitive inhibition:

$$v = V / [(1 + K_m^{\text{app}}/s)(1 + i/K_i^{\text{app}})] \quad (6)$$

in which:

$$K_m^{\text{app}} = K_m(1 + H/K_1) \quad (7)$$

and

$$K_i^{\text{app}} = K_i(1 + K_2/H) \quad (8)$$

The secondary plots in Figs. 2 and 5 were based on eqns. (7) and (8) respectively. In Fig. 2(b) the intercept on the vertical axis gives K_m and the intercept on the horizontal axis gives $-K_1$. Correspondingly, in Fig. 5(b) K_i and $-1/K_2$ are obtained from the intercepts on the vertical and horizontal axes respectively.

Temperature-dependence of the K_m and pK values

Fig. 6 shows some results of K_m determinations at various temperatures. With rising temperatures the K_m values are apt to be decreased, the mean change being 0.35 mM/1°C. The ΔH value of the

Fig. 7. van't Hoff plot relating pK to temperature

The pK values of the ionizable group affecting the K_m^{app} of pantothenase were determined at different temperatures as described in the text. The ΔH value of H^+ ion binding estimated from the slope of the line is -59 kJ/mol .

substrate binding was $+15 \text{ kJ/mol}$ when calculated on the basis of K_m values.

The temperature-dependence of the pK value (and particularly the derived ΔH value) of the ionizable group in the active centre is suggested as a reliable indicator of the character of the ionizable group (Dixon & Webb, 1964). Fig. 7 presents the van't Hoff plot for the pK values of pantothenase between 9° and 24°C . The ΔH value of the H^+ ion binding is -59 kJ/mol as derived from the Figure.

K_m values, which are dependent on temperature, are required when the pK values are to be calculated from eqn. (3). On the other hand, the K_m^{app} values obtained from the measurements are dependent on the pK values (i.e. on K_1), as shown by eqn. (7). The ΔH values derived from Figs. 6 and 7 cannot thus be regarded as very accurate. To obtain precise values of ΔH , the K_m values rather than the K_m^{app} values should be determined at various temperatures. The temperature-induced changes of K_m and K_1 can also be calculated on the basis of the information supplied in Figs. 6 and 7, by using eqns. (3) and (7) and the van't Hoff equation in the following way.

Eqn. (3) can be rearranged to $K = L/(1 + s/K_m)$ where $L = (H_1 v_j - H_i v_i)/(v_i - v_j)$. At two different temperatures T_1 (K_1 , L_1 , K_{m1}) and T_2 (K_2 , L_2 , K_{m2}) eqn. (9) is obtained:

$$\frac{K_1}{K_2} = \frac{L_1 (1 + s/K_{m2})}{L_2 (1 + s/K_{m1})} \quad (9)$$

The task is to estimate K_1/K_2 (or ΔH) so that eqn. (9) is valid. The term L_1/L_2 on the right-hand

side of eqn. (9) can be derived directly from Fig. 7. $L_1/L_2 = K_1/K_2$ because K_m was constant on calculation of the values in Fig. 7. K_m values in the term $(1 + s/K_{m2})/(1 + s/K_{m1})$ are derived from eqn. (7): $K_m = K_m^{app}/(1 + s/K)$; K_m^{app} values come from Fig. 6, and K values are derived from the van't Hoff equation:

$$\ln \left(\frac{K_1}{K_2} \right) = -\frac{\Delta H}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

if a suitable estimated value is given to ΔH . This ΔH is best chosen near the ΔH from Fig. 7. Thus values both to the left-hand side and to the right-hand side of eqn. (9) can be calculated for each estimated ΔH value. Then the values are plotted against the estimated ΔH , and the ΔH at which the values are equal is the final correct ΔH . These calculations will alter the substrate-binding ΔH of pantothenase to -14 kJ/mol , and the H^+ ion-binding ΔH to -50 kJ/mol . These values are likely to better represent the real values than the values ($+15$ and -59 kJ/mol) derived directly from the Figures.

Reaction with phenylmethanesulphonyl fluoride

Phenylmethanesulphonyl fluoride is used as an $-OH$ reagent particularly to trace serine in enzymes (Fahrney & Gold, 1963). The serine proteases are typical phenylmethanesulphonyl fluoride-inhibited enzymes. Kinetic similarities between serine proteases and pantothenase induced the author also to test a possible inactivation of pantothenase with this inhibitor. Pantothenase was inactivated at 10°C with half-times of 50 min in Mops buffer and 130 min in phosphate buffer.

Discussion

The kinetic equation presented above was formed on the basis of the assumption that the substrate and the inhibitor have separate binding sites in the enzyme. This agrees well with the fact that inhibition is non-competitive; a coinciding binding site would result in a competitive inhibition. Good evidence of the validity of the kinetic pattern is provided by its capability of describing the behaviour of the enzyme at various pH values, in various buffers, and in the presence of various inhibitors. The formation of the equation was based on the assumption that the ligand bindings should have no effects on each other. This assumption may not, however, be entirely valid because a lower value for the binding constant is obtained from the protecting ability of oxalate against thermal inactivation than from inhibition, the former value thus being measured in the absence of substrate (R. K. Airas unpublished results). Nor have

effects of phosphate been taken into consideration in the equation. In the presence of phosphate the enzyme has a lower K_m value, and a slightly dissimilar curve v versus pH. Some interaction between the substrate binding and the phosphate binding seems to prevail since a lower pK value is obtained on the basis of the K_m values than from the curve v versus pH; in the former measurement the substrate concentration is varied, in the latter it is constant.

The ΔH value of the ligand binding may be affected by both the actual binding and the simultaneous changes in the protein molecule. The ΔH of the binding of a mere small-molecular ligand is low, positive or negative (Steinhardt & Reynolds, 1969). The ΔH values of the conformational changes of the proteins, on the other hand, are generally above 100 kJ/mol (Table 16 in Tanford, 1968; Garnier, 1966; Atanasov *et al.*, 1968; Jackson & Brandts, 1970; Pohl, 1968; Mazhul *et al.*, 1970). A high ΔH of a ligand binding suggests a conformational change in the protein. The ΔH value, 50 kJ/mol, of the ionizable group at the pantothenase substrate-binding site is comparatively high, but it need not signify any simultaneous changes in the structure of the protein. The pK value 7.0 as well as the ΔH value 50 kJ/mol suggest a histidine imidazole. In free amino acid the pK is 6.0 and ΔH is 30 kJ/mol; in ribonuclease, for instance, the pK values of imidazole range from 5.7 to 6.8 and the ΔH values from 17 to 88 kJ/mol (Roberts *et al.*, 1969).

The bond in pantothenate broken by pantothenase is an amide bond, in principle similar to the peptide bond broken by proteolytic enzymes. The peptidases, particularly the so-called serine proteases (chymotrypsin and subtilisin, for instance) have in their active centres a histidine residue whose imidazole reveals a somewhat elevated pK value; also the serine proteases are active at the alkaline side of the

pK value (Blow & Steitz, 1970). Similar properties in the structures of the active centres are further suggested by the inactivation of both pantothenase and serine proteases by phenylmethanesulphonyl fluoride.

To judge from the pK value, the ionizable group at the binding site of the inhibitor is apparently a histidine imidazole. An inhibitor with a negative charge will then be bound to the imidazole form with a positive charge.

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