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Activation enthalpies and pH dependence of phenol hydroxylase from *Trichosporon cutaneum*, in vitro and in situ

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The effect of pH and temperature on phenol hydroxylase in vitro was compared to the corresponding effect on the enzyme in situ, in permeabilized cells. Activation enthalpies in situ were about 75–80% of those in vitro, in both cases decreasing with increasing pH (6.0–8.5). The order of addition of phenol and NADPH affected the K_m values for phenol at 25°C, but not at 10°C. The results support the idea that the enzyme in situ is in a more favourable position for catalysis than the purified enzyme and that slow conformational changes, triggered by binding of phenol, become rate limiting above 10°C.

Phenol hydroxylase; Kinetics; Activation enthalpy; Phenol binding; (*Trichosporon cutaneum*)

1. INTRODUCTION

Phenol hydroxylase, isolated from the soil yeast *Trichosporon cutaneum* catalyzes the reaction:

$$\text{phenol} + O_2 + \text{NADPH} + H^+ \longrightarrow \text{catechol} + \text{NADP}^+ + H_2O$$

The enzyme is dimeric ($M_r = 2 \times 76000$) and contains two non-covalently bound FAD per dimer [1,2]. Besides being a substrate, phenol acts as an inhibitor [3,4] and also as an effector, causing conformational changes, with concomitant changes in the affinity towards NADPH [3] and FAD [5], in the reactivity of essential amino acid residues [2,6] and in the FAD spectrum of the enzyme [3]. Slow conformational changes occur during the overall reaction of phenol hydroxylase [7]. Such changes are much slower with the enzyme in situ than with the enzyme in vitro, the kinetics with all known substrates of phenol hydroxylase being biphasic [4]. The present paper deals with the effects of temperature and pH on the activity of phenol hydroxylase, comparing the purified enzyme with the enzyme in situ, in permeabilized cells, with respect to enthalpies of activation and kinetic constants.

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EDTA, ethylene diaminetetraacetate
Enzymes: phenol hydroxylase (EC 1.14.13.7); catechol 1,2-oxygenase (EC 1.13.1.1)

2. MATERIALS AND METHODS

2.1. *Chemicals*

All chemicals were reagent grade commercial preparations. Most of them were purchased from Sigma Chemicals (St. Louis, MO), except inorganic salts and phenol, which came from Merck (Darmstadt, FRG).

2.2. *Enzyme, enzyme assays, protein determinations and determination of enzyme-phenol dissociation constants*

Phenol hydroxylase was induced, isolated and assayed essentially as described earlier [1,2,8]. Measurements of reaction velocities in situ and in vitro were made by the oxygen reduction assay with a Clark oxygen electrode [4]. The assays in vitro were with 0.1–0.3 mg purified enzyme protein, those in situ with 6.0 mg total protein in permeabilized cells. Spectra were recorded in a Cary 219 spectrophotometer. Dissociation constants of

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enzyme-phenol complexes were determined by spectrophotometric titrations with phenol, essentially as described earlier [5]. The concentrations of purified enzyme were calculated from the extinction at 442 nm of enzyme-bound FAD (12.6 mM^{-1}). Concentrations in the range 5.6–16.6 μM of enzyme-bound FAD were used. Perturbations of the absorption spectra by phenol were measured as difference spectra. The $\Delta A_{478-458}$ values were used to compute the concentration of enzyme-phenol complexes and of free phenol. Buffers used were Mes-KOH (pH 6.0 to 6.5), Hepes-KOH (pH 7.0 to 7.5), Bicine (pH 8.0 to 8.5) and Na-borate (pH 9.0). The kinetic analysis was performed on a HP-85 desk computer using the non-linear algorithm of Marquardt [9,10].

2.3. *Enthalpies of activation*

The energy of activation, E_a, was determined from Arrhenius plots of log v versus $1/T$, where v is the initial reaction rate and T is the absolute temperature. At least two measurements were performed at each temperature. The least square's method was used to give the best estimation of the slope of the lines. The correlation factor, r, was calculated as:

$$r = \frac{n\Sigma xy - \Sigma x \Sigma y}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2] \cdot [n\Sigma y^2 - (\Sigma y)^2]}}$$

Activation enthalpies, ΔH_a were calculated as $\Delta H_a = E_a - RT$ (where R is the gas constant) according to the transition-state theory [11].

2.4. *Kinetic parameters*

Half saturation constants for the overall reaction in situ and in vitro, for simplicity called $K_{m(phenol)}$, are formally derived from the Michaelis-Menten equation. However, the term 'half saturation constant' is preferred because of the biphasic kinetics of phenol hydroxylase. Dissociation constants of enzyme-phenol complexes using purified phenol hydroxylase are defined as:

$$K_{d(phenol)} = \frac{[\text{enzyme}] \times [\text{phenol}]}{[\text{enzyme} - \text{phenol}]}$$

3. RESULTS

3.1. *The temperature dependence of the initial rate of the overall reaction of phenol hydroxylase in vitro and in situ at pH 6.5, 7.5 and 8.5*

With the purified enzyme, the reaction rates increased with increasing temperature up to 30°C, at all three pH values (fig.1). With the enzyme in situ, broad temperature optima were observed, instead. These were between 22 and 30°C at pH 7.5 and 8.5, but between 28 and 37°C at pH 6.5 (fig.1).

Arrhenius plots were constructed for the enzyme in vitro and in situ at selected pH values in the range 6.0 to 8.5, using saturating phenol concentrations. Linear plots were obtained in the temperature range 10–25°C in situ and 10–30°C in vitro.

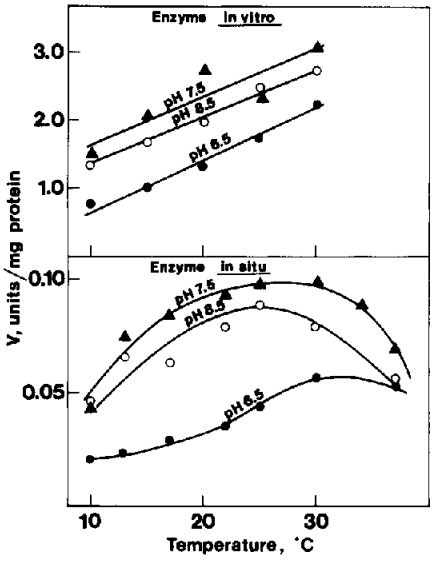

Fig.1. The pH and temperature dependence of the overall reaction of phenol hydroxylase in vitro and in situ. Measurements were performed as described in section 2, spectrophotometrically in vitro and with the oxygen reduction assay in situ. Phenol concentration: 0.17 mM in vitro, 0.1 mM in situ. Values plotted are the means of at least two measurements.

Table 1 shows that the activation enthalpies deduced from these plots decreased with increasing pH values, both in vitro and in situ. At all the pH values examined, the activation enthalpies in situ were about 75–80% of those in vitro. Statistical

Table 1

The pH dependence of enthalpies of activation (ΔH_a) for phenol hydroxylase in vitro and in situ

pH	Enzyme in vitro		Enzyme in situ	
	ΔH_a	r	ΔH_a	r
6.5	50.5	0.96	40.4	0.94
7.0	36.6	0.97	nd	
7.5	31.2	0.96	24.3	0.95
8.0	30.0	0.94	nd	
8.5	25.6	0.94	19.1	0.91

Conditions as in fig.1. Calculations of ΔH_a and the correlation coefficient r are as in section 2. nd, not determined. ΔH_a given in $kJ \cdot mol^{-1}$

evaluation of the data indicates significance of the observed differences (table 1).

3.2. *The dependence of $K_{m(phenol)}$ on the order of addition of phenol and NADPH at 10°C and 25°C, respectively*

The order of addition of phenol and NADPH did not affect the values of $K_{m(phenol)}$ at 10°C. This is in contrast to what was observed at 25°C, when addition of phenol prior to NADPH resulted in half saturation constants for phenol which were several times lower than when NADPH was added first (fig.2). When measured in situ, in permeabilized cells at 10°C and pH 7.6, the $K_{m(phenol)}$ showed a value of 7.5 μM when phenol was added first and 8.6 μM when NADPH was added first (not shown), thus only slightly higher. Previously, we have shown that this difference was much greater at 25°C, viz. 0.15 μM when phenol was added first and 6.8 μM when NADPH was added first [4]. V_{max} values at each pH were not affected by the order of addition of phenol and NADPH, whether in situ or in vitro.

3.3. *The pH and temperature dependence of $K_{d(phenol)}$ and $K_{m(phenol)}$ for purified phenol hydroxylase*

At 10°C, the $K_{d(phenol)}$ decreased with increasing pH (fig.2). A corresponding study at 25°C is necessarily subject to some uncertainty, because the enzyme is not very stable at that high temperature during the prolonged time (several hours) required for spectrophotometric titration with phenol. It could be concluded, however, that the $K_{d(phenol)}$ values in the pH range 7.5–9.7 were considerably lower at 25°C than those observed at 10°C. At 10°C, the $K_{m(phenol)}$ had a pronounced maximum around pH 7.5, whereas no maximum was observed at 25°C, the K_m values having a tendency to increase with increasing pH (fig.2).

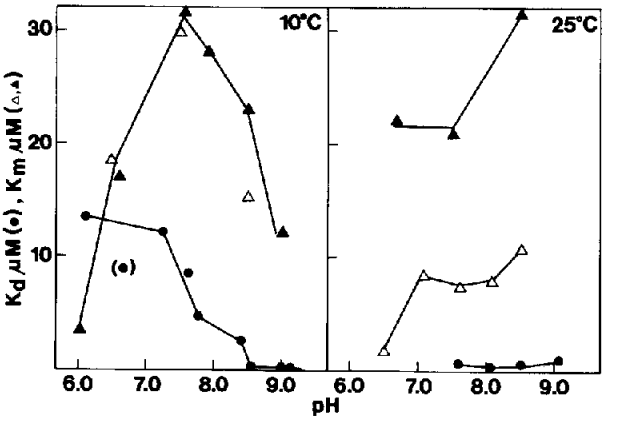

Fig.2. The dependence of $K_{m(phenol)}$ and $K_{d(phenol)}$ on pH and on the order of addition of phenol and NADPH to purified phenol hydroxylase. The $K_{m(phenol)}$ values were obtained from measurements by the oxygen reduction assay, using 0.1–0.3 mg purified enzyme. The enzyme was equilibrated with either phenol or NADPH for 2 min, before start of the overall reaction. (•) $K_{d(phenol)}$; (△) $K_{m(phenol)}$, phenol added first; (▲) $K_{m(phenol)}$, NADPH added first.

4. DISCUSSION

It is reasonable to assume that the conditions for enzyme assays in situ are closer to those in vivo than when the assays are carried out with purified enzymes, in vitro. This pertains at least to the local protein concentration and to interactions with macromolecular cell components. There are reports in the literature on different behaviour of enzymes when measured in situ and in vitro [12,13]. We have reported earlier that the half saturation constant for phenol is much lower with phenol hydroxylase in situ than with the purified enzyme, at least when measured at optimum pH [4]. The present results show that the phenomenon occurs throughout a broad pH range. It also occurs with a second enzyme of the phenol degradative pathway, viz. catechol 1,2-oxygenase, for which K_m at pH 8.3 is 3.3 μM in situ, but 5.9 μM in vitro [14].

The difference between phenol hydroxylase in situ and the enzyme in vitro extends to energetics of the reaction, the activation enthalpies for phenol hydroxylase in situ being about 75–80% of those in vitro (table 1). This indicates that the enzyme in situ is in a more favourable situation for catalysis than the purified enzyme. There are no data in literature on activation enthalpies for aromatic hydroxylases, that can be used for comparison with phenol hydroxylase.

In a salt medium containing phenol, the temperature optimum for growth of *T. cutaneum* is around 28°C. This correlates with the temperature optimum for the enzyme in situ, but not with the activity of the purified enzyme, which increases steadily, at least up to 30°C (fig.1).

The catalytic cycle of phenol hydroxylase, a three-substrate enzyme, consists of a reductive and an oxidative phase comprising at least 10 steps. With resorcinol as a model substrate, three short-lived intermediates (I, II, III) are postulated for the oxidative phase [15]. The conversion of I to II and II to III is acid catalyzed, whereas the conversion of III to the oxidized enzyme is base catalyzed, the latter step being rate limiting at pH 7.6 [16], which is the optimum pH for the overall reaction. The decrease of the activation enthalpy for the overall reaction with phenol with increasing pH (table 1) correlates with these findings.

At 10°C, the $K_{d(phenol)}$ and the $K_{m(phenol)}$ differ in their pH dependence (fig.2). This indicates that the value of $K_{m(phenol)}$ is not governed by the binding of phenol alone, but by subsequent steps in the reaction sequence, as well. At 10°C, the order of addition of phenol and NADPH did not affect the K_m values for phenol, either in vitro or in situ, whereas at 25°C the addition of phenol prior to NADPH resulted in half saturation constants which were several times lower than when NADPH was added first (fig.2). It has been postulated that binding of phenol triggers slow conformational changes in the enzyme, which affects the initial rate of the overall reaction [7]. When the overall reaction rate slows down at 10°C, the slow conformational changes may no longer be rate limiting. This could explain why $K_{m(phenol)}$ was not significantly affected by the order of addition of phenol and NADPH at 10°C, while it was affected at 25°C.

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