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# IN SITU AND IN VITRO KINETICS OF PHENOL HYDROXYLASE

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SUMMARY. The half saturation constant for phenol was much lower with phenol hydroxylase in situ than with the purified enzyme, whereas the constant for NADPH was higher. In both cases, the linearized plots of the Michaelis-Menten equation were biphasic and the half saturation constants for all phenolic substrates were several times lower, when the phenol was added to the assay medium before NADPH, than when NADPH was added first. There was a similar, but much smaller, effect on the half saturation constants for NADPH. The V-values were not affected by the order of addition. The results suggest slow conformational changes in the enzyme during the overall reaction, which seem even slower, when the enzyme is measured in situ. 

The dimeric enzyme, phenol hydroxylase from the soil yeast Trichosporon cutaneum (EC 1.14.13.7), is a monooxygenase containing 2 FAD per 2x76 Kdal and employing NADPH as an electron-donating cosubstrate (1-3). The enzyme undergoes profound changes upon binding of phenol, as reflected in perturbation of the flavin absorption spectrum, quenching of flavin fluorescence, strength of FAD-attachment, reactivity of certain amino acid residues (2-5) and cooperativity of phenol-binding sites (6). There are indications of slow, thiol-modulated changes during the overall reaction (Neujahr, in preparation). The present investigation was undertaken to study the effect of such slow changes on kinetic parameters of the overall reaction with phenol and phenol analogues and to compare some of these effects between purified enzyme and the enzyme in situ, in permeabilized cells. In situ measurements come much closer to the physiological conditions in vivo, than do measurements in vitro. One important factor is the actual concentration of the enzyme, which can be several orders of magnitude higher in vivo than those employed with purified enzymes (7-9). Polyethylene glycol

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(PEG) decreases the activity of water available in an enzyme solution, thus increasing the true concentration of the enzyme. This may, under certain conditions, mimick the situation of the enzyme *in situ* as well as that *in vivo*. We have therefore determined kinetic parameters of phenol hydroxylase also in the presence of PEG.

EXPERIMENTAL. All chemicals were reagent grade commercial preparations, whenever available, and purchased as before (5,12). Phenol hydroxylase was induced and isolated from T.cutaneum, essentially as described earlier (1), with slight modifications. Enzyme units (U) are defined as µmoles $O_2$ reduced or NADPH oxidized per min. Protein concentration was determined according to Bradford (10) or computed from absorption spectra.

Assay of phenol hydroxylase and determination of kinetic parameters. A mixture of seven purified enzyme preparations was incubated with 1 mM dithiothreitol, 0.1 mM EDTA and 10 µM FAD for 2 hours at 4°C and then passed through a G25 Sephadex column equilibrated with potassium phosphate 0.1 M pH 7.6, containing the same additions. The eluate, containing 19 enzyme units (4.7 mg protein) per ml, was diluted to 10 U/ml and frozen in 0.9 ml portions, each sufficient for one experimental series. Phenol hydroxylase *in situ* was assayed in cells from fully induced cultures on phenol (1), permeabilized with 0.05% Triton X-100 by the method of Miozzari et al. (11) as modified by Mörtberg and Neujahr (12). Measurement of reaction velocities was made by the oxygen reduction assay with a Clark Oxygen Electrode, Model YSI 4004 (Yellow Spring Instrument Co., Yellow Spring, OH). The assays were made at 25°C in 1.7 ml air-saturated HEPES 0.05 M pH 7.6, containing 1 mM dithiothreitol, 0.1 mM EDTA, 10 µM FAD, 500-1000 U catalase (EC 1.11.1.6) to eliminate the $H_2O_2$ formed in a side reaction of phenol hydroxylase (3) and 70 µg purified enzyme protein or 230-460 µg protein as permeabilized cells. Kinetic parameters of the enzyme were determined by numerical analysis, using the non-linear regression algoritm of Marquardt (13) adapted for computers according to Nash (14). Three types of kinetic parameters were determined, *viz* half saturation constants (K), maximum initial rates (V) and substrate inhibition constants ($K_i$). The K- and V-values were formally derived from the Michaelis-Menten equation, the $K_i$-value from the equation

$$\frac{v}{V} = \frac{[S]}{K + [S] + [S]/K_i}$$

RESULTS AND DISCUSSION. The effect of the order of addition of phenol and NADPH on kinetic parameters of purified phenol hydroxylase. Table 1 lists half saturation constants for several phenol derivatives which act as substrate-effectors of phenol hydroxylase (3). With all the phenols tested, these constants were several times lower, when the phenol was added before NADPH than when NADPH was added first. The K-value for catechol was as

Table 1. Kinetic parameters of purified phenol hydroxylase in the overall reaction with various phenols depending on the order of addition of phenol and NADPH

| Phenolic substrate | K (phenol) | | $K_i$ | K (NADPH) | |
|---|---|---|---|---|---|
| | Phenol first | NADPH first[1)] | Phenol first | Phenol first | NADPH first[1)] |
| | μM | μM | μM | μM | μM |
| Phenol | 7.3 | 21 | 720 | 40 | 50 |
| Catecnol | 0.6 | 12 | 12500 | 72 | 90 |
| Resorcinol | 9.8 | 32 | 1800 | 53 | 50 |
| o-Fluorophenol | 0.6 | 5 | 30800 | 23 | 40 |
| m-Fluorophenol | 1.4 | 8 | 6300 | 19 | 30 |
| p-Fluorophenol | 5.4 | 17 | 2300 | 34 | 100 |
| o-Chlorophenol | nd | nd | nd | 250 | 700 |
| m-Chlorophenol | 4.3 | 55 | 2300 | 130 | 600 |
| p-Chlorophenol | 3.8 | 39 | 2600 | 130 | 500 |
| o-Methylphenol | nd | nd | nd | 1100 | 1300 |
| m-Methylphenol | 2.4 | 31 | nd | 810 | 700 |
| p-Methylphenol | 5.8 | 21 | nd | 520 | 800 |

nd = not determined, due to extremely slow reaction rates
1) From Ref 3, selected controls confirmed these values.
Oxygen reduction assay as described in EXPERIMENTAL. The concentration of NADPH was 2 mM while varying the concentrations of phenol, catechol, resorcinol and fluorophenols, and 5 mM while varying the concentrations of chloro- and methylphenols. With variable concentrations of NADPH, the levels of the respective phenols were kept at 0.15 mM. The standard deviations of the listed half saturation constants (K) were in the range 10-25%.

much as 20 times lower when catechol was added before NADPH than when NADPH was added first. With most of the other phenol derivatives, the difference was 6-13 times, whereas with phenol, resorcinol, p-fluorophenol and p-methylphenol, it was 3-4 times. With all phenol derivatives tested, the $K_i$-values were significantly higher than with phenol itself, with o-fluorophenol more than 40 times nigher. The half saturation constants for NADPH with various phenol derivatives were affected to a lesser degree by the order of addition of the phenol and NADPH, but they were, in many cases, significantly lower when the phenolic substrate was added first. The V-values were only slightly affected by the order of addition of phenol and NADPH (data not shown). These results suggest that slow equilibria occur during the overall reaction of phenol hydroxylase.

Comparison of the kinetics of purified phenol hydroxylase with those of the enzyme in situ

Table 2 shows that also with the enzyme in situ, the addition of phenol prior to NADPH gave half saturation constants for phenol which were lower (ca 45 times) than when NADPH was added first,

Table 2. The effect of the order of addition of phenol and NADPH on the kinetic parameters of phenol hydroxylase, when assayed in permeabilized cells as compared to purified enzyme. Conditions as in Table 1, but using 3.4 mM NADPH in the presence of PEG; phenol was 75 µM while varying NADPH. Standard deviations from the K-values were in the range 20-25%, those from the V-values around 5%. K and $K_i$ are listed as µM, V as units/mg.

| Enzyme and additional assay conditions | Phenol | | | NADPH | |
|---|---|---|---|---|---|
| | K | V/K | $K_i$ | K | V/K |
| . Purified enzyme phenol first, 2 min | 7.3 | 0.49 | 720 | 40 | 0.095 |
| . Purified enzyme NADPH first, 2 min | 21 | 0.19 | nd | 50 | 0.072 |
| . Purified enzyme in 10% PEG 400 phenol first, 2 min | 9.3 | 0.34 | 2500 | nd | nd |
| . Permeabilized cells phenol first, 2 min | 0.15 | 0.87 | 7200 | 1600 | $1.3 \times 10^{-4}$ |
| . Permeabilized cells NADPH first, 2 min | 6.8 | 0.02 | nd | nd | nd |
| . " " 5 min | nd | nd | nd | 670 | $2.4 \times 10^{-4}$ |

nd = not determined

the respective V-values not being significantly affected. Irrespective of the order of addition of phenol and NADPH, there were large differences between the purified enzyme and the enzyme *in situ*. With phenol added first, the K-value for phenol was 7.3 µM for the purified enzyme, but only 0.1-0.5 µM for the enzyme *in situ*. With NADPH added first, the difference was smaller, but still pronounced, 21 µM phenol for the purified enzyme and 6.8 µM for the enzyme *in situ*. The effect of the order of addition of phenol and NADPH on the K-values for NADPH was smaller with both, the purified enzyme and with the enzyme *in situ*. However, in contrast to the K-values for phenol, those for NADPH were much higher for the enzyme *in situ* than for the purified enzyme (Table 2). These observations suggest that the slow conformation changes during the overall reaction are much slower *in situ* than with the purified enzyme.

The effect of PEG on the activity of purified phenol hydroxylase

There was a slight increase of the initial rates of phenol hydroxylase reaction when the standard assay mixture contained 10% PEG 400, whereas PEG 2000 was entirely without effect. However, the data in Table 2 show that the presence of 10% PEG 400 did not significantly affect the K- or the V-value for phenol. On the other hand, the inhibition by excess phenol was largely affected by PEG, the $K_i$ for the purified enzyme

increasing from 720 µM phenol in the absence of PEG to 2500 µM in its presence. The $K_i$ measured in situ was still higher, 7200 µM.

The biphasic kinetics of phenol hydroxylase

The Michaelis-Menten treatment of initial rate data offers a convenient way of comparing parameters for various substrates also in cases when the enzyme does not entirely follows the Michaelis-Menten mechanism. This is the case with phenol hydroxylase. The kinetic parameters for the reaction of the enzyme with phenol and several phenol derivatives, estimated earlier (3) and reassessed with respect to the order of addition of phenolic substrate and NADPH in the present investigation (Table 1), are all calculated from Michaelis-Menten and substrate inhibition equations. The range of substrate concentrations that was used for the original estimation of kinetic parameters of phenol hydroxylase (3) gave good fits to various linearized plots derived from the Michaelis-Menten equation. This was because the lower limit of assayed phenol and NADPH concentrations was about 5 µM (3). Similar lower levels were also employed in studies on phenolic hydroxylases by other workers (15-16). In the present investigation, the range of assayed phenol concentrations was extended to very low values, 1 µM and below. This revealed the biphasic (or multiphasic?) nature of the kinetics of phenol hydroxylase. Biphasic Eadie-Hoffstee plots for phenol, catechol and resorcinol are shown in Fig 1. Similar biphasic kinetics were observed with

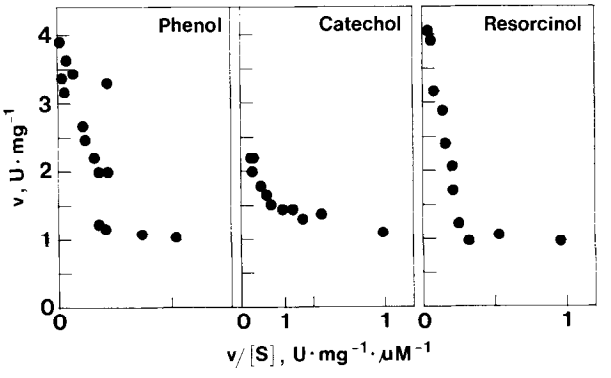


Fig 1. Eadie-Hoffstee plots of the initial rates (v) of phenol hydroxylase with phenol, catechol and resorcinol, added to the enzyme before NADPH. Assay conditions as in Table 1. Similar biphasic plots were obtained with practically all the phenolic substrates listed in Table 1.

practically all phenolic substrates that were tested, using phenol as well as NADPH as the variable substrate, with the purified enzyme and also when the enzyme was assayed in the presence of PEG or in situ. It thus appears that "biphasic kinetics" is an inherent property of the reaction mechanism of phenol hydroxylase. The biphasic kinetics corroborates our earlier observations on the occurrence of cooperativity during binding of phenol to phenol hydroxylase (6) and of slow equilibria during its overall reaction.

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