Kinetics of fungal catalases inhibition with hydroxylamine

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Inhibition of the catalase from *Aspergilus niger* was performed with hydroxylamine (HA). The inhibition decreased at acid pH with pK_a 6.0 indicating that only the unprotonated form of HA acts as an inhibitor. Hydrazine (HZ), with its structure very similar to that of HA, does not inhibit catalase even in unprotonated state. At HA more than 1 µM, biphasic kinetics of hydrogen peroxide splitting was observed. HA induced generation of a new absorbance band in presence of hydrogen peroxide. Dialysis of the reaction mixture indicated the reversible character of HA action. Kinetic and spectral investigations give evidence of HA interaction with compound I of catalase.

Key words: catalase, *Aspergillus niger*, hydroxylamine, inhibition

INTRODUCTION

The catalases are involved in the neutralization of hydrogen peroxide in the cells. The literature data show that catalases isolated from animals, plants and microorganisms are similar; they are composed of four identical subunits with a native molecule mass in the range of 225–270 kDa, contain one protoporhyrin IX group/subunit, exhibit a broad pH range (5–10) [1, 2]. Due to universal spreading of catalases they are the common impurities of microbial enzymes. In some cases, for example, in application of oxidases for analytical purposes, the impurities of catalases may be vital due their high activity [3].

The catalases show a high specificity to hydrogen peroxide, and only some compounds inhibit their activity. Azide and cyanide act as irreversible inhibitors [4]. The catalases from mammalians are irreversibly inhibited by 3-amino-1,2,4-triazole (AT) in presence of hydrogen peroxide [5, 6]. Inhibition of catalase with AT is associated with protein modification [1, 7].

Very recently the phenomenology of fungal catalases inhibition with hydroxylamine has been described [8]. The aim of the current work was to investigate the kinetics of inhibition of *A. niger* catalase with hydroxylamine and related compounds.

MATERIALS AND METHODS

The catalase from *A. niger* was received from Novozymes A/S (Denmark). The concentration of catalases was determined spectrophotometrically using the absorbance ε_{280} = 150 mM^{-1} cm^{-1}. Hydrogen peroxide solution was prepared in water from 30% Perhydrol (Reachim, Russia) and the concentration was determined spectrophotometrically using the extinction coefficient 39.4 M^{-1} cm^{-1} at λ 240 nm. Hydrazine hydrate (HZ) was purchased from Bayer, hydroxylamine sulfate (HA), potassium dihydrophosphate and potassium chloride were received from Reachim (Russia). Experiments were performed in 10 mM phosphate buffer solutions containing 0.1 M of KCl, pH 7.2. All solutions were prepared from chemical-grade reagents and in 3 times distilled water.

Spectrophotometric measurements were performed by using a computer-assisted Gilford Instrument 2600 spectrophotometer and an Otsuka RA-401 stopped-flow spectrophotometer interfaced with computer systems.

Catalase activity in steady state conditions was measured by using a home-made computer-assisted hydrogen peroxide electrode in a 50 ml cell at 30 °C. The current of 6.0 µA corresponded to 1 mM of

hydrogen peroxide. Fast kinetics was performed in rapid scan mode at wavelength range 385–460 nm at 30 °C in phosphate buffer solution pH 7.2. The concentration of catalase was 2.8 μM, hydrogen peroxide was 10 μM, hydroxylamine concentration changed in the interval 0.03–4.0 mM.

Long-term dialysis was performed according to the following procedure. The activity and spectra of the catalase were recorded in the beginning at 360–480 nm. After addition of the inhibitor and hydrogen peroxide the activity and spectra of the mixture were recorded again. The reaction mixture was applied to a dialysis tube and was dialyzed against the cooled buffer solution (500 ml) in the refrigerator by stirring. The activity of the catalase was measured at respective time by withdrawing the aliquot (0.08 ml) of the mixture in presence of 1 mM of hydrogen peroxide.

RESULTS

The activity of the catalase (1 nM) was linearly proportional to hydrogen peroxide concentration (0.05–1.1 mM). Addition of 1 μM of HA to the reaction mixture decreased the enzyme activity 5.5 times. The pH dependence of HA inhibition showed that the inhibition decreased in the acid area and the pK_a of transition was 6.0 ± 0.1. This indicated that only the unprotonated form of HA acted as an inhibitor. No catalase inhibition was indicated at pH 7.2 up to 0.2 mM of hydrazine. Investigation of inhibition at pH 7–9 did not reveal any inhibition even at pH larger than pK_a of hydrazine, which was equal to 7.95.

The dependence of catalase inhibition on HA concentration showed a typical competitive inhibition if the reaction rate was 0.7–1.7 min (Fig. 1). However, it was indicated that the kinetics of hydrogen splitting was biphasic with a rather low inhibition at the beginning of the process (Fig. 2). Changing the HA concentration little influenced the initial rate, but inhibited the rate at the second phase (Fig. 2).

Rapid scan measurements indicated that the absorbance of the catalase changed only if HA was mixed with hydrogen peroxide (Fig. 3). The absorbance decreased at 410 nm and increased at 426 nm with formation of an isobestic point at 421 nm.

Changes in enzyme activity and spectrum after addition of HA and hydrogen peroxide were investigated by term dialysis. After addition of hydrogen peroxide to the catalase–HA mixture the enzyme activity sharply decreased (Fig. 4). However, the activity slowly increased during 4 hours of dialysis and reached 93% of the initial value. The spectrum of the catalase–HA mixture also changed and was similar to the spectrum recorded in rapid scan experiments (Fig. 3). During dialysis the spectrum of

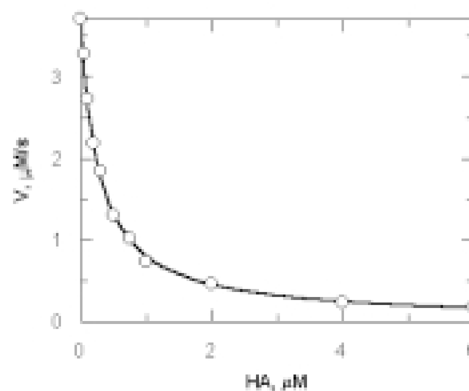


Fig. 1. Dependence of catalase activity on HA concentration at the second phase of inhibition. 10 mM phosphate buffer solutions containing 0.1 M of KCl, pH 7.2, at 30 °C. Concentrations: catalase 2.6 nM, H_2O_2 1.0 mM, HA 0 – 6.0 μM

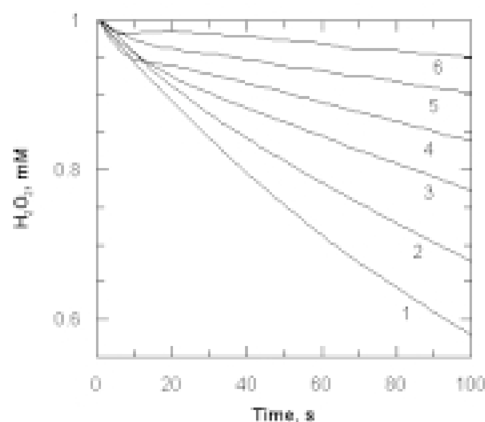


Fig. 2. Kinetics of *A. niger* catalase-catalysed hydrogen peroxide splitting at different HA concentrations. Concentrations HA: 0 μM (1), 0.1 μM (2), 0.2 μM (3), 0.5 μM (4), 1.0 μM (5), 2.0 μM (6), other conditions as in Fig. 1

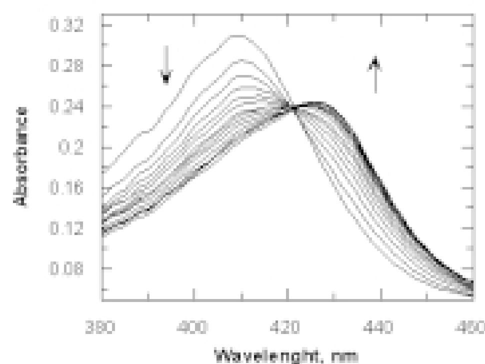


Fig. 3. Absorbance of *A. niger* catalase during reaction with H_2O_2 and HA. 10 mM phosphate buffer solutions containing 0.1 M of KCl, pH 7.2, at 30 °C. The spectra were recorded at 20 ms. Concentrations: catalase 2.8 μM, H_2O_2 10 μM, HA 2 mM

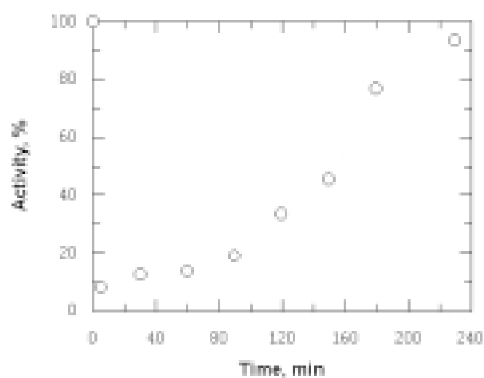
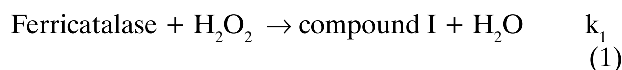


Fig. 4. Change of *A. niger* catalase activity during dialysis. 10 mM phosphate buffer solutions containing 0.1 M of KCl, pH 7.2, at 30 °C. Initial concentration of the reagents in the dialysis tube were: catalase 2.8 μM, HA 4 mM, H_2O_2 10 μM

this mixture changed and after 4 hour of dialysis returned to its initial state (data not shown).

DISCUSSION

To explain the experimental facts, a scheme of HA action was suggested. The essence of HA action is a slow formation of the complex [compound I/HA], which slowly decomposes to ferricatalase and product, possibly through compound II:



$$\text{Ferricatalase} + H_2O_2 \rightarrow \text{compound I} + H_2O \qquad k_1 \qquad (1)$$

$$\text{compound I} + H_2O_2 \rightarrow \text{ferricatalase} + O_2 + H_2O \qquad k_2 \qquad (2)$$

$$\text{compound I} + \text{HA} \rightarrow [\text{compound I/HA}] \qquad k_3 \qquad (3)$$

$$[\text{compound I/HA}] \rightarrow \text{compound I} + \text{HA} \qquad k_4 \qquad (4)$$

$$[\text{compound I/HA}] \rightarrow \text{ferricatalase} + \text{product} \qquad k_5 \qquad (5)$$

The system of differential equations corresponding to this scheme was solved by the adaptive Runge–Kutta method. The H_2O_2 decomposition rate constants k_1 and k_2 were calculated from the initial rate dependence of H_2O_2 concentration. Following this scheme, the inhibition was calculated at the second quasi-stationary phase and compared with the experiment at 0.7–1.7 min (Fig. 1). The variation coefficient of calculated and experimentally determined inhibition was 4.3%, showing a good compliance of the model with the real HA action. The calculated constants were: k_1 = 1.4 $\mu M^{-1}s^{-1}$, k_2 = 5.6 $\mu M^{-1}s^{-1}$, k_3 = 0.82 $\mu M^{-1}s^{-1}$, k_4 = 0.0062 s^{-1}, k_5 = 0.02 s^{-1}. K_i calculated as k_4/k_3 was 0.0076 μM.

The kinetic measurements did not allow to make conclusions on the mechanism of catalase turnover through HA. It is possible that during the turnover compound II was formed, too. The spectrum of the intermediate is very similar to the absorbance of compound II of peroxidases. If compound II was regenerated, the calculated constant k_5 corresponded to compound II reduction with HA.

ACKNOWLEDGEMENTS

Authors express sincere thanks to Palle Schneider (Novozymes A/S, Denmark) for donating the catalase and for permanent interest and useful discussions during this work.

Received 14 October 2003

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KATALAZĖS IŠ *ASPERGILLUS NIGER* INHIBICIJA HIDROKSILAMINU

S a n t r a u k a

Katalazės iš *A. niger* inhibicija buvo atliekama su hidroksilaminu (HA). Inhibicija sumažėja esant rūgštiniam pH, kai pK_a 6,0. Tai rodo, kad tik neprotonuota HA forma veikia kaip inhibitorius. Hidrazinas (HZ), kurio struktūra panaši į HA, neinhibuoja katalazės netgi neprotonuotos būsenos. Esant daugiau nei 1 μM HA buvo stebima bifazinė vandenilio peroksido skaldymo kinetika. HA indukuoja naujos sugerties atsiradimą esant vandenilio peroksidui. Reakcijos mišinio dializė rodė grįžtamąjį HA veikimo pobūdį. Kinetiniai ir spektriniai tyrimai patvirtino akivaizdžią HA sąveiką su katalazės tarpiniu junginiu „compound I“.