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Horseradish peroxidase inhibition by thiouracils

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Abstract In this paper, the activity of horseradish peroxidase was further determined in the presence of several uracil derivatives. The rate of guaiacol peroxidation decreases in presence of 2-thiouracil and of 6-*n*-propyl-2-thiouracil, but is not changed by 6-*n*-propyluracil nor uracil. Thus, thiouracils inhibit horseradish peroxidase in a noncompetitive form. The binding of 6-*n*-propyl-2-thiouracil, 2-thiouracil, 6-*n*-propyluracil and uracil with horseradish peroxidase shows difference spectra due to changes in the environment of heme group in peroxidase. Then, the binding sites for these uracil derivatives are in an hydrophobic pocket at the heme periphery of peroxidase. The lesser binding rates were for uracil and propyluracil, which did not inhibit the peroxidase activity. These results point to the thiol group in uracils as responsible for the inhibition of peroxidase activity through interaction with an allosteric binding site, in peroxidase heme environment.

Key words: Horseradish peroxidase inhibition; Thiouracils binding; Difference spectrophotometry; Catalitic binding site; 6-*n*-Propyl-2-thiouracil; Uracil derivative

1. Introduction

Horseradish peroxidase, HRP, a 42-kDa glycoprotein containing a single protohemin, is a typical member of the plant peroxidases that serve as bactericides [1,2]. In common with many other heme peroxidases, it is oxidized by two equivalents by peroxides, and it carries out one-electron oxidation on a number of aromatic substrates. HRP can also carry out a two-electron oxidation of some inorganic ions such as I^- and SCN^- which bind to HRP only at very acidic pH [3–5]. In addition to the native substrates, phenols and aromatic amines, HRP can bind a range of structurally related substrates or substrate analogs [1,3,6–8]. Elegant suicide inhibitor studies using phenylhydrazine have revealed that the phenyl group is incorporated into the chromophore solely at the δ-meso position [9], supporting a binding pocket at (and direct electron transfer via) the heme periphery between pyrroles A and D [10]. The binding of these donor molecules and competitive inhibitors occurs in the vicinity of the heme peripheral 8-methyl group, with hydrophobic interactions probably with Tyr-185 and with hydrogen bond with adjacent amino acid residues such as Arg-183 [11].

The 6-propyl-2-thiouracil (PTU), an antithyroid drug, decreases the activity of thyroid iodide peroxidase [12], myeloperoxidase and eosinophil peroxidase [13]. The PTU treatment reduced the electron donor specificity of the latter peroxidases. Although PTU leads to a change in the structure surrounding the heme iron of myeloperoxidase and eosinophil peroxidase [13] and a structural alteration of HRP [14], its mechanism of inhibition still remains to be elucidated. The purpose of the present paper is the determination of the inhibition mechanism of HRP by this thiouracil.

2. Materials and methods

2.1. Products

Horseradish peroxidase (EC 1.11.1.7, donor H_2O_2 oxidoreductase), uracil, 2-thiouracil, 6-*n*-propyl-2-thiouracil and guaiacol were purchased from Sigma Chemical Co. (St. Louis, USA). Ethylenglycol (ethanodiol), ethanol and hydrogen peroxide were purchased from Merck. 6-*n*-Propyluracil was synthesized according to the method of Lindsay et al. [15]. Proteins and uracils were dissolved in 66.7 mM phosphate buffer pH 7.4. The HRP concentration was determined optically at 403 nm using the molar absorptivity coefficient ($\varepsilon^{1\%}$) 1.02×10^5 $cm^{-1} \cdot M^{-1}$ [16].

2.2. Equipment

A double beam UV-VIS spectrophotometer model 3600 from Beckman was used. This spectrophotometer was completed with a thermostatic bath from Selecta (Spain), which has been used to maintain a constant temperature of 20°C during the experiments. Double compartment quartz cuvettes, with a 0.4375×2 cm lightpath, from Hellma (Germany), permitted us to perform directly the difference spectra. Equilibrium dialyses were carried out with a Dianorm dialyzer (Diachema AG, Zürich), spinning at 8 rpm. Data analysis was performed with the Enzfitter iterative program from Biosoft (UK) at a Tandon computer.

2.3. Measurements of peroxidase activity (Guaiacol assay)

Assay of HRP activity was carried out by the measurement of guaiacol peroxidation [12]. The assay system contained 0.9 ml of guaiacol in 66.7 mM phosphate buffer, pH 7.4, and 0.025 ml of 10^{-8} M enzyme solution. The reaction was started by the addition of 0.05 ml of 12 mM hydrogen peroxide. The concentration of synthesized tetraguaiacol was determined by the absorbancy changes at 470 nm using a molar absorptivity coefficient, ε, of 2.66×10^4 $cm^{-1} \cdot M^{-1}$. Guaiacol concentrations between 0.3 and 30 mM were used. The peroxidase activity was also measured in the presence of the inhibitor (uracil). The inhibitor concentration was between 2.7×10^{-10} and 10^{-9} M. HRP was preincubated with the inhibitor for 15 min.

2.4. Measurement of binding (difference absorbance)

The binding measures were made by the difference absorbance technique, previously described [17]. In one of the compartments of the reference cuvette we placed the HRP and in the other compartment the uracil. The sample cuvette was filled with the HRP–uracil blend in one compartment and the buffer solution in the other. From the increase in absorbance, ΔA, at the wavelength of the maximum absorbance in the difference spectra, $\lambda_{max} = 410$ nm, we calculated the concentration of chromophore binding (C_b):

$$C_b = \Delta A/(\varepsilon_b - \varepsilon_f) \times 1$$

Starting from the spectra of every uracil, the molar extinction coefficients, ε_f, were calculated. HRP concentrations of 2.5×10^{-5} M mix with the uracil concentrations between 0 and 1.00×10^{-5} M were used in the ε_b estimation. The light path was that in the difference spectra ($l = 0.4375$ cm).

On the other hand, in order to characterize the nature of the differ-

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ence spectra obtained, spectra of the uracils dissolved in ethanol at 50% and at several pHs were carried out.

3. Results and discussion

Uracil and 6-*n*-propyluracil do not affect the peroxidase activity. However, the rate of guaiacol peroxidation decreases in presence of 2-thiouracil (TU) and of 6-*n*-propyl-2-thiouracil (PTU). As can be seen in the Lineweaver–Burk plots for HRP activity (Fig. 1), the TU and PTU inhibit the peroxidase activity at several donor molecule (guaiacol) concentrations. The catalytic constant, K_{cat} (5×10^5 min^{-1}), is reduced to the half ($K'_{cat} = 2.5 \times 10^5$ min^{-1}) in the presence of stoichiometric concentrations of TU or of PTU. K_M (7×10^{-3} M) is not affected by the presence of these thiouracils. These results show that the thiouracils assayed inhibit the HRP activity in a noncompetitive form with the donor molecule. Thus, at stoichiometric concentrations of HRP and inhibitor, this inactivates half of the HRP molecules. This effect may be explained by an equimolar equilibrium between the inhibitor free and bound on HRP.

Since difference spectroscopy permits the detection of small changes in the environment of a chromophore and the study of ligand binding to proteins [17], we have used this technique to quantify the thiouracil binding and detect the chromophores interacting in the HRP–uracil binding.

Interactions of PTU, TU, 6-*n*-propyluracil and uracil with HRP lead to formation of complexes that exhibit an absorption band with the maximum at 408 nm. This band is shifted about 5 nm towards the red region in comparison to the spectrum of HRP (λ_{max} = 403 nm). As a result of this, difference absorption spectra appear which are characterized by a positive extreme at 410 nm, a negative extreme at 400 nm and an isosbestic point at 404 nm. Dissolving HRP in ethylenglycol produced a similar difference spectrum (Fig. 2). Since ethylenglycol decreases the polarity of the solvent, the spectra result from the movement of the HRP molecule from an aqueous to a more hydrophobic environment. The HRP chromophore at 403 nm is the heme group. Then, all these uracils interact hydrophobically with the heme group of the peroxidase.

From the spectra of the HRP, extinction coefficients at 410 nm of $\varepsilon_f = 87600$ $cm^{-1} \cdot M^{-1}$ and $\varepsilon_b = 90600$ $cm^{-1} \cdot M^{-1}$ were obtained. These values were used in the calculation of the heme bound in the HRP–uracils complexes. The maximum binding rate obtained for the thiouracils (PTU and TU) are 0.336 mol of heme bound per mol of protein, whereas a maximum binding rate of 0.1 mol of heme bound per mol of HRP was found for 6-*n*-propyluracil and uracil (molar ratio of the uracil/HRP = 20). Then, HRP binds all the studied uracils, with lesser

Table 1
Heme bound to PTU, TU, PU and U when the HRP concentration was 5.00×10^{-5} M

Uracil/HRP	[Heme] bound (M)			
	PTU	TU	PU	U
0.5	5.54×10^{-6}	4.35×10^{-6}	3.95×10^{-6}	2.37×10^{-6}
1.0	6.72×10^{-6}	5.54×10^{-6}	4.35×10^{-6}	3.16×10^{-6}
2.0	7.91×10^{-6}	6.72×10^{-6}	4.75×10^{-6}	3.95×10^{-6}
3.0	9.01×10^{-6}	7.91×10^{-6}	5.14×10^{-6}	4.74×10^{-6}
4.0	1.03×10^{-5}	9.49×10^{-6}	5.54×10^{-6}	5.14×10^{-6}
5.0	1.15×10^{-5}	1.15×10^{-5}	5.73×10^{-6}	5.54×10^{-6}

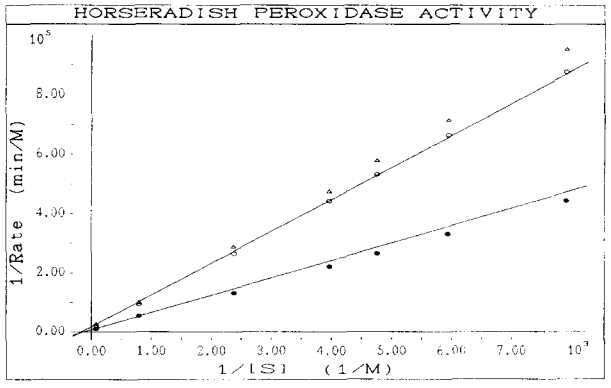

Fig. 1. Lineweaver–Burk plots for the peroxidase activity of HRP (●); and of HRP in the presence of stoichiometric concentrations of PTU (○) or of TU (△). The concentration of HRP was 2.6×10^{-10} M.

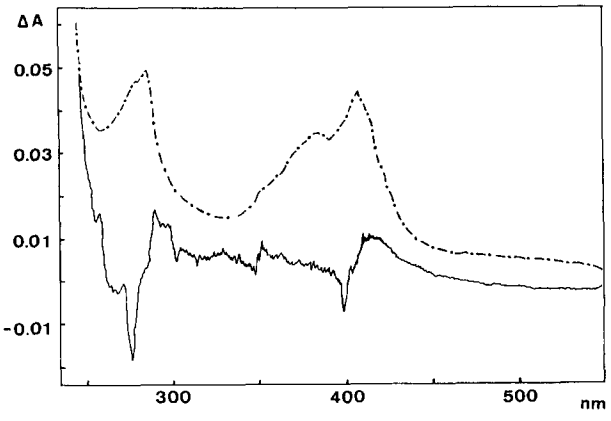

Fig. 2. Difference absorption spectra of HRP in 2.5×10^{-5} M PTU (––) and in 20% ethylenglycol (-·-). The concentration of HRP was 2.5×10^{-5} M.

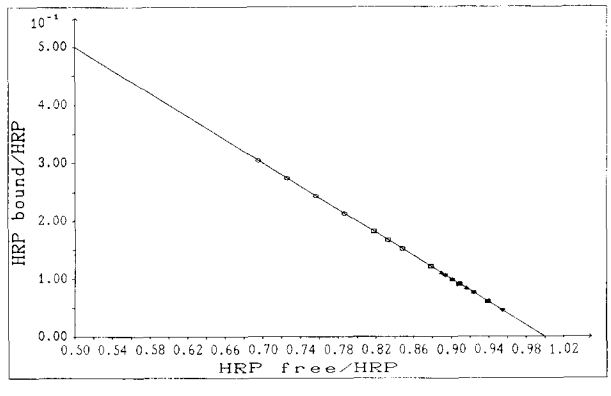

Fig. 3. Ratios of HRP bound to PTU (○), TU (□), 6-*n*-propyluracil (▲) and uracil (▼). The total HRP concentrations were 1.25×10^{-5} M (○,□) and 5.00×10^{-5} M (▲,▼).

binding capacity for 6-*n*-propyluracil and uracil (Table 1 and Fig. 3). The HRP saturation could be overtaken (Fig. 3).

The HRP activity measurements show that only the uracil derivatives with a thiol group inhibit the peroxidase activity. Uracil and 6-*n*-propyluracil do not affect the peroxidase activity. However, all uracils bind on the heme group of HRP. Since

the lesser ability to bind TU-HRP and PTU-HRP, the interactions of uracils with the environment of the heme group could be responsible for the peroxidase inhibition by thiouracils. But also, the thiol group interacting with the apoprotein would alter the spectrum of the heme by perturbing its environment without directly interacting with the heme. This latter is in accordance with the structural change in HRP previously observed by dilatometry and viscometry [18] and can explain the noncompetitive inhibition by an allosteric effect of thiouracils on HRP.

In summary, HRP binds all the uracils but is inhibited only by thiouracils (non-competitively). The non-competitive inhibition occurs by the binding of uracils on the heme group in HRP at the non-catalytic site of the enzyme. The thiol group in thiouracils causes the structural change, previously observed.

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