Horseradish Peroxidase-Catalyzed Oxidation of Rifampicin: Reaction Rate Enhancement by Co-oxidation with Anti-inflammatory Drugs

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The tuberculostatic drug rifampicin has been described as a scavenger of reactive species. Additionally, the recent demonstration that oral therapy with a complex of rifampicin and horseradish peroxidase (HRP) was more effective than rifampicin alone, in an animal model of experimental leprosy, suggested the importance of redox reactions involving rifampicin and their relevance to the mechanism of action. Hence, we studied the oxidation of rifampicin catalyzed by HRP, since this enzyme may represent the prototype of peroxidation-mediated reactions. We found that the antibiotic is efficiently oxidized and that rifampicin-quinone is the product, in a reaction dependent on both HRP and hydrogen peroxide. The steady-state kinetic constants K_m^{app} (101±23 μmol/l), V_{max}^{app} (0.78±0.09 μmol/l·s^{-1}) and k_{cat} (5.1±0.6 s^{-1}) were measured (n=4). The reaction rate was increased by the addition of co-substrates such as tetramethylbenzidine, salicylic acid, 5-aminosalicylic acid and paracetamol. This effect was explained by invoking an electron-transfer mechanism by which these drugs acted as mediators of rifampicin oxidation. We suggested that this drug interaction might be important at the inflammatory site.

Key words tuberculosis; rifampicin; horseradish peroxidase; rifampicin-quinone; paracetamol

It is well-established that a peroxidase-mediated reaction is involved in the activation of the prodrug isoniazid. Its antituberculosis activity requires *in vivo* activation by KatG, a bacterial enzyme with dual activities of catalase and peroxidase. In fact, it is widely accepted that the antibiotic resistance of some strains of *Mycobacterium tuberculosis* is in part due to their reduced KatG catalase-peroxidase activity.[1] Rifampicin is a broad-spectrum antibiotic and a key component in antituberculosis therapy, whose mechanism of action lies in its high capacity to bind and inhibit the bacterial RNA polymerase.[2] However, there is evidence that redox reactions involving rifampicin also play an important part in the mechanism of action. In this connection, leukocytes from cerebrospinal fluid of rabbits infected intracisternally with *S. pneumoniae* and treated with rifampicin produced less reactive oxygen species (ROS) than did leukocytes from animals receiving β-lactam antibiotics.[3] The level of malondialdehyde in cerebrospinal fluid, an indication of oxidative stress, was also lower in rifampicin-treated animals.[3] These data corroborate the fact that rifampicin scavenges ROS produced by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-activated neutrophils.[4] It has also been described as an immunosuppressive drug,[5] and it was shown recently that rifampicin and analogues have the potential to block tumour necrosis factor (TNF)- or phorbol myristate acetate (PMA)-induced, nuclear factor kappaB (NF-kB) activation in the Jurkat T-cell line.[6] It is a reasonable conjecture that these immunomodulatory features of rifampicin might be linked to its major oxidation product, the rifampicin quinone, since this compound was described as the real immunosuppressive agent and not the rifampicin itself.[7] In fact, it may be the case that the rifampicin quinone acts on NF-kB activation, since this transcription factor is highly sensitive to redox-active substances and the quinone moiety is very susceptible to electron transfer reactions.[8,9]

The proposal that rifampicin-quinone may be responsible for the immunosuppressive properties of rifampicin, together with the involvement of peroxidation reactions both at inflammatory sites and in the metabolism of mycobacteria, led us to study the mechanism of rifampicin oxidation catalyzed by horseradish peroxidase (HRP). This peroxidase was chosen since it is a prototype of peroxidation catalysts. A classical steady-state kinetic study was performed and K_m and K_{cat} were determined. The role of other tuberculostatics and nonsteroidal anti-inflammatory drugs (NSAID) as co-substrates during rifampicin oxidation was evaluated. We found that rifampicin was an effective substrate of HRP and that it is oxidized by a classical peroxidation mechanism, in which the reduction of compound-II by rifampicin is the rate-limiting step. The reaction rate can be increased considerably by the presence of other substrates of peroxidase, including NSAID. Paracetamol was a very efficient activator of the oxidation.

MATERIALS AND METHODS

Chemicals Hydrogen peroxide 30% (w/w) was purchased from Peroxidos do Brazil (São Paulo, SP, Brazil). Horseradish peroxidase (EC 1.11.1.7 type IV), 3,3′,5,5′-tetramethylbenzidine (TMB), isoniazid, rifampicin, pyrazinamide, L-ascorbic acid and Ficoll-Hypaque 1077 and 1119 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Paracetamol, diclofenac, 5-aminosalicylic acid, salicylic acid, piroxicam and dipyrone were purchased from Purifarma (São Paulo, SP, Brazil). All the reagents used for buffer preparation were of analytical grade.

Solutions Hydrogen peroxide was prepared by diluting a 30% stock solution and its concentration was calculated from its absorption at 240 nm (ε=43.6 mol^{-1}·l·cm^{-1}).[10] HRP stock solutions were prepared in water and its concentration was calculated from its absorption at 403 nm (ε=1.03×10^5 mol^{-1}·l·cm^{-1}).[11] Rifampicin stock solution was prepared in 10% dimethylformamide aqueous solution.

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TMB stock solution was prepared in dimethylformamide. The absorption spectra and initial reaction rates were measured in a Diode Array spectrophotometer (Hewlett Packard 8452A).

RESULTS

We demonstrated that HRP was able to catalyze rifampicin oxidation by following the changes in the absorption spectrum during the reaction course (Fig. 1A). The formation of peaks with distinct isosbestic points at 307, 357, 422 and 521 nm indicates that a unique product was formed in the reaction. As would be expected from the oxidation of the hydroquinone functional group of rifampicin, the product of the reaction showed a visible spectrum (absorption band centered at 550 nm) that matched that of its rifampicin-quinone derivative, which may be obtained by chemical autoxidation or HRP-mediated aerobic oxidation.[12,13] In fact, Kono have demonstrated that HRP-catalyzed rifampicin oxidation can be performed in the absence of hydrogen peroxide, but in this case the reaction is very slow, taking about 90 min for total conversion of the rifampicin (50 μmol/l).[13] The presence of the quinone group was confirmed by its reduction back to the hydroquinone form when ascorbic acid was added to the reaction medium (Fig. 1B). Indeed, ascorbic acid is frequently used to keep this tuberculostatic in its reduced form.[14]

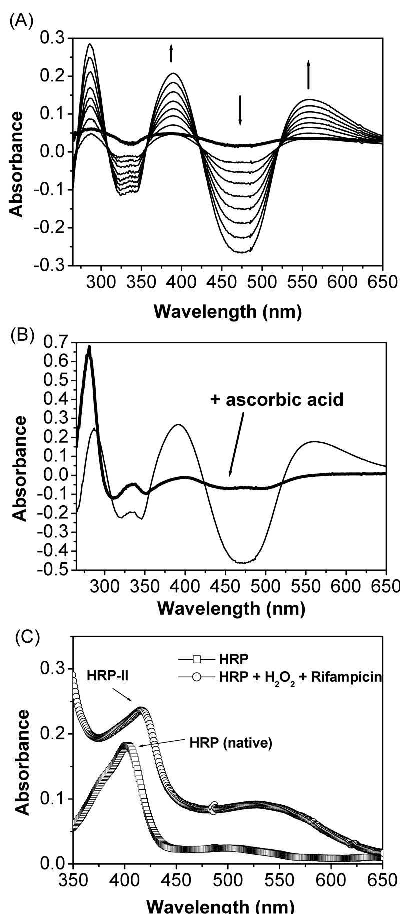


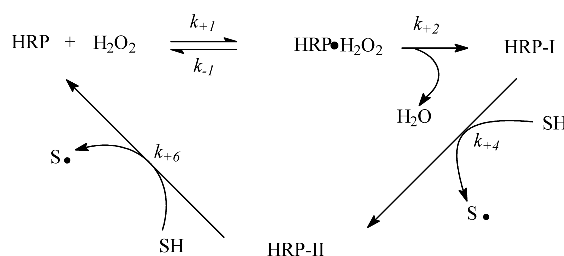
Fig. 1. Oxidation of Rifampicin Catalyzed by HRP

(A) Difference spectra against a rifampicin blank were recorded each 10 s after addition of H_2O_2. The experimental conditions were: Rifampicin 100 μmol/l, H_2O_2 200 μmol/l, HRP 10 nmol/l in 80 mmol/l phosphate buffer, pH 5.4 at 25 °C. The reactions were triggered by adding H_2O_2. (B) The reduction of oxidized rifampicin (rifampicin quinone) by ascorbic acid. After complete oxidation, 200 μmol/l of ascorbic acid was added to the reaction medium. (C) The spectra of native HRP and HRP-II. The latter was present during the reaction course. The experimental conditions were the same, except HRP, which was 1 μmol/l.

The bleaching of the visible band of rifampicin at 472 nm was chosen to monitor the reaction rate. As occurs in most HRP-catalyzed reactions, the rate of oxidation of rifampicin increases in acid medium.[11] Here we worked at pH 5.4, as it is typically used for peroxidase-catalyzed reactions. Neither the temperature nor the types or concentrations of ions in the buffer were important for the reaction efficiency (data not shown). By following the changes in the heme Soret absorption region of the HRP spectrum, we found that the rate-limiting step for rifampicin oxidation was the reduction of compound II of HRP (HRP-II, band at 418 nm), this intermediate being the only form detected spectroscopically during the course of the reaction (Fig. 1C).

In the classical peroxidation mechanism, the native enzyme is oxidized by hydrogen peroxide, generating a redox intermediate known as compound I (HRP-I). This two-electron-oxidized form of HRP is able to oxidize many different molecules through one-electron transfer, being reduced in the process to compound II (HRP-II). This one-electron-oxidized form of HRP oxidizes less strongly, but can still act upon many molecules, being reduced to the original native form (Chart 1).[11] Recently, a steady-state mathematical model was proposed for the kinetics of reactions that obey this mechanism under conditions approaching substrate saturation (Eqs. 1—3 in Chart 1).[15,16] Since the oxidation of rifampicin matches these criteria, we used this model to determine the turnover number (k_{cat}) for its oxidation.

The change in absorbance at 472 nm ($\varepsilon = 15400 \cdot \mathrm{M}^{-1} \cdot \mathrm{cm}^{-1}$)



$$V_o = \frac{V_{max}^{app}[SH]_o}{K_m^{app} + [SH]_o} \quad (1)$$

$$V_{max}^{app} = \frac{2k_{cat}[H_2O_2]_o[HRP]_o}{(k_{cat}/k_{+1}) + [H_2O_2]_o} \quad (2)$$

$$k_{cat} = \frac{k_2 k_6}{k_2 + k_6} \quad (3)$$

where: HRP represents the native form of the enzyme

HRP-I represents the redox intermediate compound I

HRP-II represents the redox intermediate compound

SH represents a substrate for HRP

S• represents the oxidized substrate

Chart 1. The Classical Peroxidation Mechanism for HRP-Catalyzed Reactions and the Equations for Steady-State Kinetic Model[12,13]

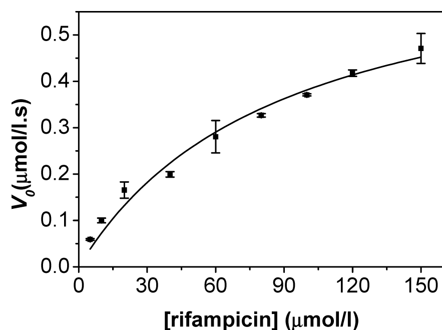


Fig. 2. Dependence of the Steady-State Initial Rate (V_o) on Rifampicin Concentration

V_o values were calculated by Δabs at 472 nm in the first minute. Except for rifampicin, the experimental conditions were as Fig. 1. The results represent the mean and S.D. of four replicates.

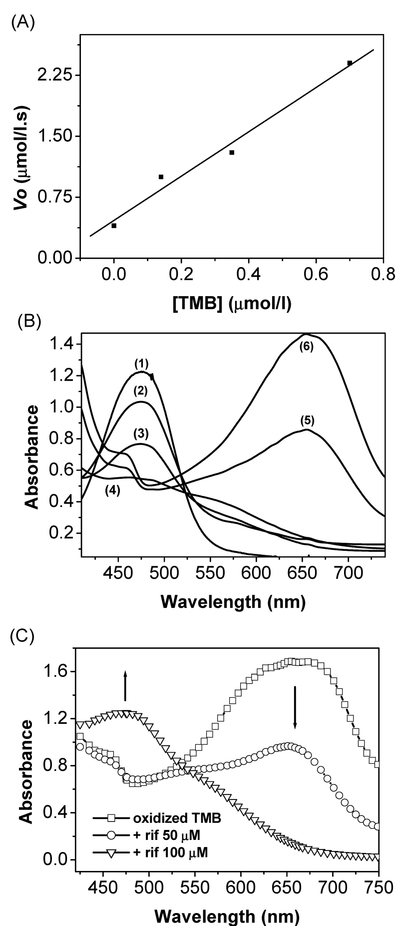


Fig. 3. Co-oxidation of Rifampicin and TMB Catalyzed by HRP

(A) Effect of TMB on oxidation of rifampicin. V_o values were calculated by Δabs at 472 nm when rifampicin concentration was kept constant and TMB concentration was varied. The experimental conditions were as Fig. 1, except for the presence of TMB. (B) Absorption spectra recorded during co-oxidation of rifampicin and TMB. The scans were recorded at (1) zero, (2) 15, (3) 30, (4) 45, (5) 60 and (6) 75 s after addition of H_2O_2.The experimental conditions were as Fig. 1, except for the presence of TMB 1.4 mmol/l. The band at 655 nm is assigned to oxidized TMB. (C) Reaction of rifampicin with oxidized TMB (1.4 mmol/l). In this case the reaction took place in the absence of rifampicin, which was added after TMB oxidation.

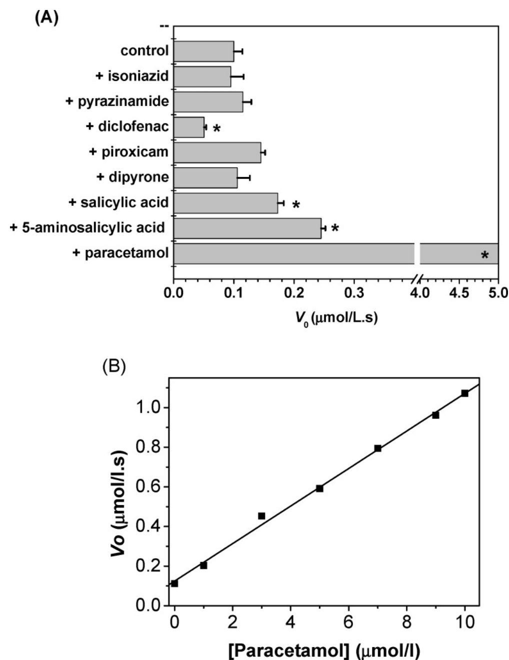


Fig. 4. Effect of Drugs on Rifampicin Oxidation

(A) Reaction rate enhancement. The experimental conditions were as Fig. 1, except for the presence of the drugs (1 mmol/l). The results represent the mean and S.D. of triplicates. Student's t-test was used to determine the difference between control and tests, with the level of significance set at $*p<0.05$. (B) Dose-dependent effect of paracetamol on HRP-catalyzed oxidation of rifampicin. The initial rates were calculated by Δabs at 472 nm when rifampicin concentration was kept constant and paracetamol concentration was varied.

over the first minute was used to obtain the initial reaction rate (V_o) while the rifampicin concentration was varied at constant hydrogen peroxide (Fig. 2). The values of the apparent Michaelis constant (K_m^{app}, $101\pm23\ s^{-1}\cdot\mu$mol/l) and apparent maximum rate V_{max}^{app} ($0.78\pm0.09\ \mu$mol/l$\cdot s^{-1}$) were obtained by nonlinear regression (Origin for Windows program, version 6.0), fitting Eq. 1 to plots of the initial rate of oxidation as a function of rifampicin concentration (Fig. 2). The turnover number k_{cat} was calculated by Eq. 2, taking the value of k_{+1}, the rate constant for formation of compound-I, to be $1.7\times10^7\ M^{-1}\cdot s^{-1}$.[12] The efficiency with which HRP catalyzes the oxidation of rifampicin, $k_{cat}=5.1\pm0.6\ s^{-1}$, was compared with that for the oxidation of tetramethylbenzidine (TMB), which is accepted as an excellent substrate for this enzyme.[17] The experimental conditions were the same as used for rifampicin and the value found for k_{cat} was $(134\pm9)\ s^{-1}$.

It has been reported that the catalytic efficiency for oxidation of some peroxidase substrates can be enhanced by the presence of a secondary substance (co-oxidation).[18,19] In confirmation of this, here we verified that the rate of rifampicin oxidation increased in a concentration-dependent manner when TMB was added to the reaction mixture (Fig. 3A). Although the HRP-catalyzed oxidation of TMB is much more efficient than that of rifampicin (see k_{cat} values above), during their co-oxidation the tuberculostatic was the first compound depleted in the mixture (Fig. 3B). These results suggested that rifampicin was reacting with the oxidized TMB, and this was confirmed by adding the rifampicin to the reaction mixture after complete oxidation of TMB (Fig. 3C).

The rate of oxidation of rifampicin was also enhanced when other oxidizable compounds were used as co-substrates. Figure 4A shows the relative increase in the reaction rate provoked by the addition of drugs, including tuberculostatics and anti-inflammatories. The effect was surprisingly high when paracetamol was used as co-substrate, as confirmed in the dose-dependent study (Fig. 4B).

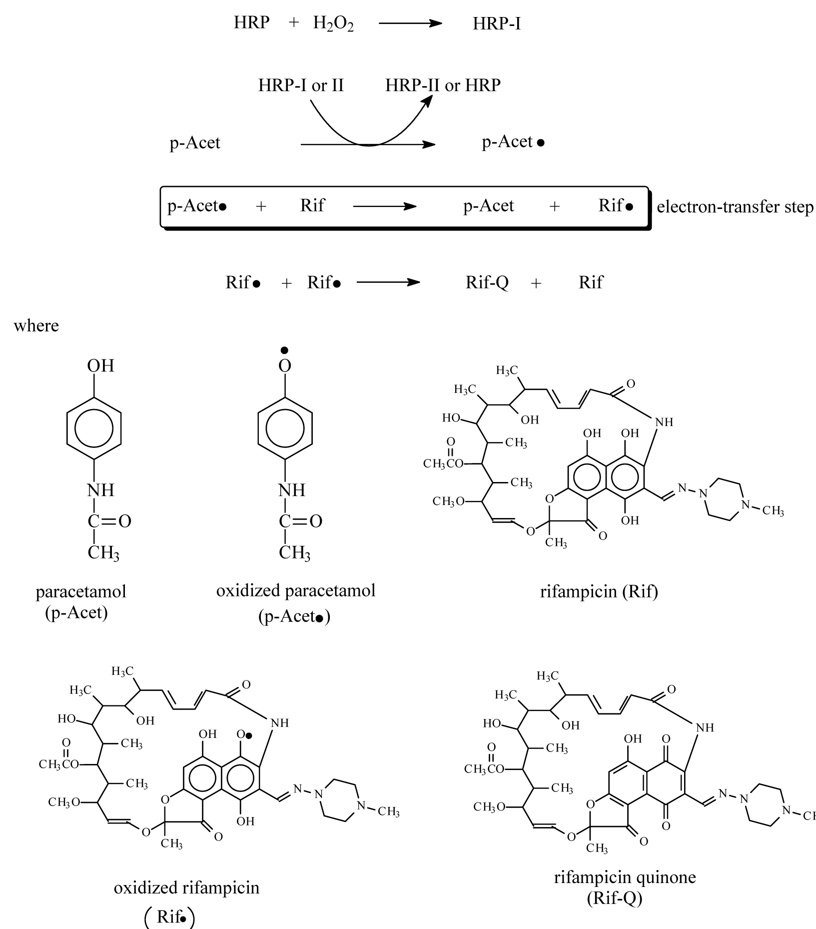


Chart 2. Proposed Mechanism for Paracetamol-Mediated HRP-Catalyzed Oxidation of Rifampicin

DISCUSSION

As might be expected, considering its polyphenolic molecular structure (Chart 2), the tuberculostatic rifampicin was oxidized in a reaction dependent on both HRP and hydrogen peroxide, both at acid and, less efficiently, at physiological pH. These results are consistent with the fact that the hydroquinone moiety found in the rifampicin molecule is a well-known substrate of peroxidases, which convert it to the quinone in a two-electron oxidation process.[20,21]

The observed increase in the rate of oxidation of rifampicin provoked by the addition of secondary substrates may be explained by the electron-transfer mechanism that has been proposed for some peroxidase-mediated reactions. For instance, hydroquinone was described as an effective activator of ascorbic acid oxidation, and chlorpromazine was found to promote the oxidation of isoniazid, in both cases by HRP-catalyzed reactions.[18,19] In such cases, the better substrate is oxidized first by the oxidized intermediate forms of peroxidases and its product is the oxidizing agent for the second substrate. This suggests that the extreme differences observed in our results between the anti-inflammatories might be related to their reactivity with HRP. This hypothesis is supported by published data about the reactivity of phenol derivatives with HRP. Indeed, Job *et al.* and Dunford *et al.* have demonstrated that phenol derivatives bearing "*para*" electron-donor substituent groups in the benzene ring, such as *p*-aminophenol, have reaction rate constants that approach the diffusion-controlled limit.[22,23] This is the case for paracetamol, the *N*-acetyl derivative of *p*-aminophenol, but not for salicylic acid or 5-aminosalicylic acid. In fact, 5-aminosalicylic acid has an electron-donor group, but in the *meta* position relative to hydroxyl (–OH). In phenol oxidation catalyzed by HRP, this small difference is quite important for the reaction rate, as can be seen from the rate constants for *p*-methoxyphenol (k_2 $1.30\times10^{8}\,\text{M}^{-1}\cdot\text{s}^{-1}$) and *m*-methoxyphenol (k_2 $2.44\times10^{6}\,\text{M}^{-1}\cdot\text{s}^{-1}$).[22] Accordingly, the present results show relative effects on oxidation of rifampicin in the following order: paracetamol>5-aminosalicylic acid>salicylic acid.

The lack of such effects with isoniazid, pyrazinamide, dipyrone and piroxicam was not unexpected, since these molecules are not as effective substrates as phenols in peroxidase-catalyzed reactions. Pyrazinamide has never been described as a peroxidase substrate. Dipyrone has been described as a thyroid peroxidase and lactoperoxidase inhibitor[24] and isoniazid, piroxicam and diclofenac as inhibitors of myeloperoxidase.[25,26] Among the drugs tested here, only diclofenac was able to inhibit significantly the oxidation of rifampicin, suggesting that diclofenac is the best inhibitor in this experimental model.

Chart 2 depicts the proposed mechanism for the oxidation of rifampicin and the role of paracetamol as a co-substrate. In this mechanism paracetamol, which is more reactive than rifampicin with compound I or II of HRP, is oxidized to its radical (*p*-Acet•). Next, in the electron-transfer step, the

paracetamol radical oxidizes the rifampicin and returns to its reduced form. Finally, the rifampicin radicals are dismutated to rifampicin-quinone and rifampicin.

These results suggest that oxidation of rifampicin is a very likely reaction at inflammatory sites. This could be the case when infectious diseases are treated with this antibiotic, since the neutrophil peroxidase and an oxidizing environment are found in such conditions. Moreover, the strong increase in the reaction rate provoked by the presence of paracetamol, which is among the commonest over-the-counter analgesic drugs, leaves little doubt that this oxidation may be a very important reaction in such a medium.

Finally, another possible and important involvement of the oxidation of rifampicin is revealed in a recent finding that oral therapy with a complex of rifampicin and HRP was more effective than rifampicin alone in an animal model of experimental leprosy. According to the authors, this drug combination produced an anti-inflammatory effect and stimulated cell immunity.[27] Considering the high reactivity of rifampicin with peroxidase, it seems likely that the quinone form of this molecule might be behind these pharmacological effects, and consequently, this issue deserves further investigation.

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