Transient-state and steady-state kinetics of the oxidation of aliphatic and aromatic thiols by horseradish peroxidase

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Abstract In the course of oxidation of thiols by peroxidases thiyl radicals are formed which are known to undergo several free-radical conjugative reactions, among others leading to hydrogen peroxide formation. The present paper for the first time presents a comparative transient-state and steady-state investigation of the reaction of 15 aliphatic and aromatic mono- and dithiols with horseradish peroxidase (HRP). Both sequential-stopped-flow spectrophotometric investigations of the reaction of HRP intermediates Compound I (k_2) and Compound II (k_3) with thiols and measurements of the overall thiol oxidation and the simultaneous oxygen consumption in the presence and absence of exogenously added hydrogen peroxide (10 μM) have been performed. With HRP as thiyl radical generator it was shown that three groups of thiols have to be distinguished: (i) Aromatic thiols (e.g. thiophenol, 2-mercaptopurine) were excellent electron donors of both Compounds (k_2: 10^4–10^7 M^{-1} s^{-1} and k_3: 10^3–10^6 M^{-1} s^{-1}); however, the overall reaction was shown to depend on addition of hydrogen peroxide, indicating insufficient peroxide regeneration by arylthiyl radicals. (ii) Aliphatic thiols which were extremely bad substrates ($k_3 < 10$ M^{-1} s^{-1}) for HRP (e.g. homocysteine, glutathione) and/or have a $pK_{a,SH} > 9.5$ (e.g. *N*-acetylcysteine, α-lipoic acid) were also shown to depend on exogenously added H_2O_2 to maintain the peroxidasic reaction, whereas (iii) with those thiols with rates of k_3 between 11 and 1600 M^{-1} s^{-1} (e.g. cysteine, cysteamine, cysteine methyl ester, cysteine ethyl ester) and/or with a $pK_{a,SH} < 8$ (penicillamine) thiol oxidation was independent of exogenously added hydrogen peroxide, indicating sufficient hydrogen peroxide regeneration.

Key words: Horseradish peroxidase; Compound I; Compound II; Thiol oxidation; Thiyl radicals; Hydrogen peroxide regeneration; Transient-state kinetics

1. Introduction

The reaction of horseradish peroxidase (HRP) with hydrogen peroxide (H_2O_2) produces a two-electron oxidized species known as Compound I [1] in which the ferric iron (Fe^{III}) is oxidized to a ferryl species ($Fe^{IV}=O$) and the porphyrin (PH) to a porphyrin radical cation ($PrxPH^{\bullet+}Fe^{IV}=O$). Stepwise reduction of Compound I by two substrate-derived electrons produces Compound II ($PrxPHFe^{IV}=O$) (in which the porphyrin radical cation has been quenched) and subsequently the resting ferric state ($PrxPHFe^{III}$). HRP and in general most of the peroxidases (EC 1.11.1.7) are known to be poorly specific for their substrates. They catalyse the oxidation of various organic and inorganic compounds such as phenols, arylamines, and halides, and with most combinations of peroxidases and substrates such oxidations have been found to proceed univalently (Reactions 2 and 3). This is also the case when thiols are oxidized. The formation of thiyl radicals has been confirmed for thiol oxidation promoted by horseradish peroxidase [2,3], lactoperoxidase [4], myeloperoxidase [5] and prostaglandin H synthase [6] by electron spin resonance (ESR) spectroscopy. Reactions 1–3 summarize the conventional peroxidase cycle with thiols (RSH) and/or thiolates (RS^-) as electron donors for Compound I and II:

$$PrxPHFe^{III} + H_2O_2 \xrightarrow{k_1} PrxPH^{\bullet+}Fe^{IV} = O + H_2O \quad (1)$$

$$PrxPH^{\bullet+}Fe^{IV} = O + RSH(RS^-) \xrightarrow{k_2} PrxPHFe^{IV} = O + RS^\bullet \quad (2)$$

$$PrxPHFe^{IV} = O + RSH(RS^-) \xrightarrow{k_3} PrxPHFe^{III} + RS^\bullet + H_2O \quad (3)$$

In contrast to the classical peroxidase substrates (e.g. phenols) and their one-electron oxidation products (e.g. phenoxy radicals), thiyl radicals show a very complex behaviour in aqueous solutions. The main conjugative reactions of thiyl radicals in aqueous solutions are conjugation with thiols or thiolates (Reaction 4) or molecular oxygen (Reaction 5) or dimerization (Reaction 6) to the corresponding disulphides [7]. These non-enzymatic free radical reactions generate superoxide radicals (Reaction 7) and by their dismutation hydrogen peroxide (Reaction 8). In consequence and in contrast to the classical peroxidase cycle, thiol oxidation is accompanied by oxygen consumption and regeneration of hydrogen peroxide:

$$RS^\bullet + RSH(RS^-) \leftrightarrow RSS(H)R^\bullet(RSSR^{\bullet-}) \quad (4)$$

$$RS^\bullet + O_2 \rightarrow RSOO^\bullet \quad (5)$$

$$RS^\bullet + RS^\bullet \rightarrow RSSR \quad (6)$$

$$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-} \quad (7)$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

Therefore the peroxidase-mediated oxidation of some thiols has been shown to be independent of addition of exogenous hydrogen peroxide [8–12] necessary for initiation and maintenance of the enzymatic cycle (Reaction 1). Nanomolar hydrogen peroxide derived from thiol autoxidation [13] has the consequence that Compound I formation is observed upon addition of these thiols to peroxidases followed by accumulation of Compound II during steady state [12].

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Characterization of the overall reaction makes it necessary to differentiate between the enzymatic (Reactions 1–3) and the non-enzymatic free radical reactions (Reactions 4–8), because the fate of thiyl radicals is strongly influenced by the kinetics of reactions that produce and remove them. But so far a comprehensive investigation of the direct reaction of thiols with HRP Compound I and Compound II (and hence of the rate of production of thiyl radicals by peroxidases) is not found in the literature. The present paper for the first time presents a sequential-stopped-flow investigation comparing 15 aliphatic and aromatic mono- and dithiols for their ability to function as electron donors for Compound I and II. In combination with steady-state measurements of the overall thiol oxidation and the simultaneous oxygen consumption in the presence and absence of exogenously added hydrogen peroxide, it was the objective not only to elucidate relationships between the structure of thiols and their abilities to react with HRP. Furthermore, it was intended to present an enzyme-based thiyl radical generator with known generation rates which offers an alternative to pulse radiolysis techniques (which so far gave the substantial part of knowledge of sulphur-centred radicals and their behaviour in aqueous solutions).

2. Materials and methods

2.1. Materials

HRP was purchased from Sigma (Type VI-A) as an essentially salt-free lyophilised powder. The RZ (Reinheitszahl, A_{403}/A_{280}) was approximately 3.0. The enzyme concentration was determined from absorbance measurements at 403 nm using a molar absorptivity of 1.0×10^5 M^{-1} cm^{-1} [14]. L-Cysteine, L-cysteamine, DL-penicillamine, DL-homocysteine, *N*-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, glutathione, coenzyme A, DL-α-lipoic acid, 2,3-dimercaptopropanol, thiophenol, 4-thiouridine, 2-mercaptopurine, 6-mercaptopurine and homovanillic acid were also purchased from Sigma. Thiol solutions were always prepared freshly and bubbled with nitrogen before use. All reactions were performed in buffers with the metal ion chelator EDTA (1 mM) which is known to prevent thiol autoxidation [13]. All other chemicals were of analytical grade. Hydrogen peroxide solutions were prepared freshly by dilution of a 30% stock solution which was purchased from Merck. Concentrations were determined spectrophotometrically at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1} [15].

2.2. Methods

2.2.1. Transient-state kinetics. The sequential stopped-flow apparatus (model SX-18MV) and the associated computer system were from Applied Photophysics (UK). For a total of 100 μl/shot into a flow cell with 1 cm light path the fastest time for mixing two solutions and recording the first data point was of the order of 1.5 ms. The enzyme concentration of the kinetic experiments was 1.7 μM HRP and the thiol concentrations were at least 10 times in excess to assure first-order kinetics. Thiol concentrations were chosen from 20 to 500 μM. Temperature was set to 25°C.

The reaction of HRP-Compound I with thiol was measured at 418 nm (Soret peak of Compound II) and 411 nm (the isosbestic wavelength between HRP-Compound II and native HRP) in the sequential stopped-flow mode. Compound I has been produced within 50 ms in the aging loop by mixing 3.4 μM HRP with 20 μM H_2O_2.

HRP-Compound II (Soret maximum at 418 nm) was produced in the aging loop by reaction of HRP with equimolar H_2O_2 and homovanillic acid concentrations at pH 10.5 in 20 mM Tris using the pH-jump technique [16]. After a delay-time of 30 s Compound II was mixed with varied concentrations of thiol (dissolved in 500 mM Tris/HCl, pH 6.5) in the ratio of 1:1 and the reaction of thiol with Compound II was measured at a resulting pH of 7.0 either at 418 nm or at 426 nm, the isosbestic point between Compound I and Compound II.

Three determinations of rate constants were performed for every thiol concentration and the mean value was used in calculation of the second-order rate constants. Each trace consisted of 400–1000 data points. Second-order rate constants were calculated from the slope of the line defined by a plot of k_{obs} versus thiol concentration.

2.2.2. Steady-state thiol oxidation. Thiol was determined according to [17] modified by Osswald et al. [10]: 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 1 mM) was dissolved in MeOH: NaOH (0.1 M) solution (98:2 $^{v}/_{v}$). The enzyme concentrations for the steady-state experiments were 17 nM to 1.7 μM HRP and the thiol concentration was 500 μM. In order to investigate the influence of hydrogen peroxide on thiol oxidation, assays were performed in the absence and in the presence of H_2O_2 (10 μM). The reaction was carried out in phosphate buffer (50 mM) containing 1 mM EDTA. Temperature was set to 30°C. After the times indicated, 100 μl of the reaction mixture were added to 900 μl of the DTNB solution. Immediately after mixing the formation of 2-nitro-5-mercaptobenzoic acid (NMBA) was quantified spectrophotometrically at 412 nm. A calibration was carried out with L-cysteine for concentrations up to 800 μM. Plots were fitted with a polynom 4th grade considering the effect of thiol-autoxidation by subtraction. The rate of thiol-oxidation mediated by HRP was calculated for the initial phase where a linear time-dependent decrease of thiol is observed.

2.2.3. Polarographic oxygen measurement. Oxygen consumption was followed polarographically (YSI 5300 Biological Oxygen Monitoring System) by using a Clark-type electrode (YSI 5331 Oxygen Probe) inserted into a stirred water bath (YSI 5301B) at 30°C. Air-saturated phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA was used. Reactions were started by addition of 17 nM to 1.7 μM horseradish peroxidase. The concentration of thiol was 500 μM. For investigation of the influence of hydrogen peroxide on thiol oxidation, reactions were performed in the absence and in the presence of H_2O_2 (10 μM). The initial rate of oxygen consumption was calculated on the assumption that a linear time-dependent oxygen consumption occurs in the initial phase of HRP-mediated thiol oxidation.

3. Results and discussion

Fifteen thiols were compared as HRP substrates in the presence and absence of hydrogen peroxide in order to elucidate structure–activity relationships of these substrates. Three dominant factors determine the reaction of thiols with HRP: (a) the actual rate of thiol oxidation by the enzyme intermediates Compounds I and II (measured directly by sequential stopped-flow spectroscopy), (b) the reactivity of thiyl radicals per se via non-enzymatic radical reactions leading to consumption of molecular oxygen (measured polarographically) and regeneration of hydrogen peroxide, and (c) pH. The influence of pH on the overall thiol oxidation has been demonstrated in previous studies [8,12]. Both thiol oxidation and oxygen consumption have been shown to increase with pH as a consequence of the increasing fraction of thiolate in the reaction medium, indicating the importance of Reaction 7 for hydrogen peroxide regeneration. The present study combines investigations of (a) and (b) at pH 7.0.

3.1. Transient-state kinetics

Fifteen thiols have been investigated for their reaction with both higher oxidation states of HRP, namely Compound I (k_2) and II (k_3). Table 1 summarizes the effects of thiol structures on both bimolecular rate constants, k_2 and k_3. Two groups of thiols have to be distinguished, namely aliphatic and aromatic ones. Compared with conventional peroxidase substrates [1] aliphatic thiols are poor electron donors for both Compound I and II. Dependent on the substituents α and β to the thiol function, rates for k_2 between 10^1 and 10^4 M^{-1} s^{-1} and between almost 0 and 10^3 M^{-1} s^{-1} for k_3 have been measured. Typical sequential stopped-flow time traces of the reactions of Compound I and Compound II with cysteine

Table 1
Summary of bimolecular rate constants for reactions of HRP Compound I (k_2) and Compound II (k_3) with aliphatic and aromatic thiols at pH 7.0

Thiol	p$K_{a,NH3+}$	p$K_{a,SH}$	Net charge at pH 7.0	% (RS–) at pH 7.0*	k_2 (mol^{-1} s^{-1})	k_3 (mol^{-1} s^{-1})
Cysteine	**10.8**	**8.3**	**0**	**5**	**220**	**11**
Cysteamine	10.8	8.6	+1	2.5	320	21
Penicillamine	10.4	7.9	0	11	24	<10
N-Acetylcysteine	–	9.5	−1	0.3	40	<10
Homocysteine	10.9	8.9	0	1.2	130	<10
Cysteine methyl ester	9.0	6.6	+0.3	72	1.3×10^4	1.6×10^3
Cysteine ethyl ester	9.2	6.7	+0.3	67	660	71
Glutathione	**9.7**	**8.8**	**−1**	**1.6**	**41**	**<10**
CoA	–	9.6	–	0.3	480	n.a.
4-Thiouridine	–	–	–	–	1.1×10^4	400
2-Mercaptopurine	–	–	–	–	**8.9×10^4**	**1.7×10^3**
6-Mercaptopurine	–	7.8	–	14	9.7×10^3	n.a.
Thiophenol	–	6.5	−0.7	76	3.28×10^7	1.2×10^6
2,3-Dimercaptopropanol	–	–	–	–	5.1×10^3	550
α-Lipoic acid	–	10.7	0	0	320	54

*The fraction of RSH in the thiolate (RS^-) form has been calculated by $[RS^-]/([RS^-]+[RSH]) = [1+10^{(pK-pH)}]^{-1}$.

The enzyme concentration was 1.7 μM HRP and temperature was set at 25°C. For details concerning sequential-stopped-flow spectroscopy, see Section 2. Data for p$K_{a,NH3}$ and p$K_{a,SH}$ are taken from [21]. Calculation of percentage thiolate at pH 7.0 is indicated.

are shown in Fig. 1(a,b). The corresponding bimolecular rates have been calculated to be 220 and 11 M^{-1} s^{-1}, respectively. Insertion of methyl groups at the carbon atom close to the mercapto group in cysteine (penicillamine) led to a 90% decrease of rate constants, whereas *N*-acetylation of cysteine (*N*-acetylcysteine) and cysteine as part of the tripeptide glutathione gave a decrease of nearly 80%. As both time traces for glutathione in Fig. 1(a,b) show clearly, these reactions are extremely slow and do not follow the pseudo-first-order kinetics exhibited by better electron donors. Above all reactions with Compound II inevitably show the inability of glutathione (as well as *N*-acetylcysteine) to reduce this enzyme intermediate. The cysteine homologue, homocysteine, showed half the activity of cysteine, whereas replacement of the carboxy group with hydrogen (cysteamine) increased the rates by about 45% compared with cysteine. However, upon esterification of cysteine a dramatic increase was observed, with the ethyl ester showing 3 times and the methyl ester showing 60 times the reactivity with Compound I compared with cysteine.

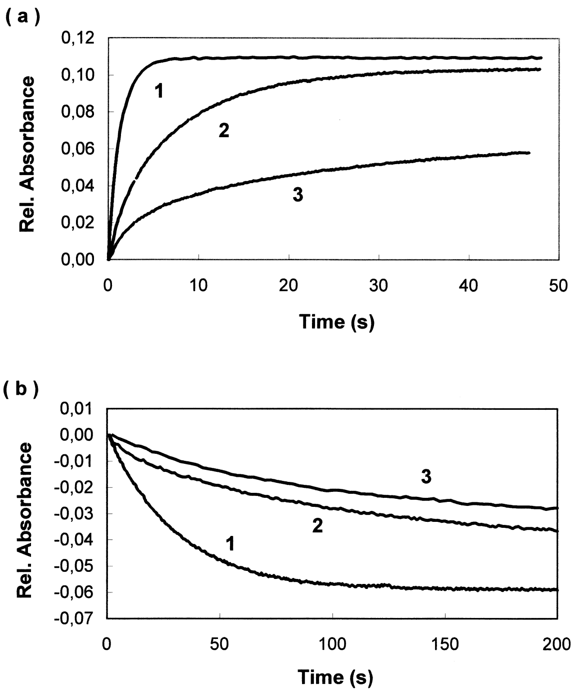

Fig. 1. Typical sequential-stopped-flow time traces for the reaction of HRP Compound I (a) and Compound II (b) with 2-mercaptopurine (1), cysteine (2) and glutathione (3). Incubation mixture contained 500 μM thiol, 1.7 μM HRP and Tris/HCl (pH 7.0). Temperature was set to 25°C. For details concerning sequential-stopped-flow spectroscopy, see Section 2.

These results indicate that both an accessible thiol and a free amino group on the neighbouring carbon are favourable for activity, which furthermore can be enhanced by replacement of the negatively charged carboxy group with hydrogen or upon esterification of it. The positive influence of a hydrophobic character on substrate binding is also demonstrated by the fact that reaction with coenzyme A was about 50% faster than with its small analogue, cysteamine. But as long as molecules with similar hydrophobic characters are compared, the influence of size becomes more important. This has been inevitably shown for cysteine methyl ester and ethyl ester and for the two dithiols investigated. Reaction rates of α-lipoic acid with Compounds I and II have been measured to be 320 and 54 M^{-1} s^{-1}, whereas with 2,3-dimercaptopropanol the reactions were accelerated by factors of 16 and 10, respectively, compared with α-lipoic acid.

In contrast to aliphatic substrates aromatic thiols showed a dramatic increase in the reaction rates. For thiophenol a k_2 of 3.28×10^7 M^{-1} s^{-1} and a k_3 of 1.2×10^6 M^{-1} s^{-1} has been measured underlining the better accessibility of the active site for aromatic compounds [18]. With 2-mercaptopurine (Fig. 1a,b), 4-thiouridine and 6-mercaptopurine the rates have been calculated to be 8.9×10^4, 1.1×10^4, and 9.7×10^3 M^{-1} s^{-1}, respectively. The keto-enol tautomeric shifts at pH 7 have been taken into account by measuring the free mercapto groups with Ellman's reagent, because it is only the free mercaptan group which is able to spend electrons to both Compound I and II. The percentage of thione forms at pH 7.0 have been calculated to be 22% (2-mercaptopurine), 81% (4-thiouridine), and 89% (6-mercaptopurine), respectively.

Table 2
Comparison of the initial steady-state oxygen consumption and thiol oxidation of both aliphatic and aromatic thiols in the absence and presence (10 μM) of hydrogen peroxide

Thiol	Initial O_2-consumption (μM O_2 min^{-1})		Initial rate of thiol oxidation (μM SH min^{-1})	
	without H_2O_2	10 μM H_2O_2	without H_2O_2	10 μM H_2O_2
Cysteine	**1.1**	**3.7**	**5.4**	**8.6**
Cysteamine	1.8	2.0	9.7	9.4
Penicillamine	5.5	4.1	15.4	11.9
N-Acetylcysteine	0.1	0.7	0.1	7.5
Homocysteine	0.6	1.8	2.8	5.6
Cysteine methyl ester	9.3	11.3	31.7	30.3
Cysteine ethyl ester	3.9	5.3	24.4	28.2
Glutathione	**0.2**	**1.5**	**1.3**	**5.0**
2-Mercaptopurine	**0.3**	**33.0**	**2.1**	**30.5**
Thiophenol	1.6	26.6	2.5	14.0
2,3-Dimercaptopropanol	2.4	2.7	6.6	10.9
α-Lipoic acid	0.5	5.2	0.2	5.9

Reactions were started by addition of HRP (1.7 μM). Incubation mixtures contained 500 μM thiol, 1 mM EDTA and 50 mM phosphate buffer, pH 7.0. Temperature was set to 30°C.

3.2. Steady-state kinetics

Without peroxidase both oxygen consumption and thiol autoxidation was less than 0.1 μM/min, indicating a nearly complete suppression of thiol autoxidation under the conditions used (see Section 2). Nevertheless, even under these conditions nanomolar concentrations of hydrogen peroxide seem to be present in order to initiate the reaction cycle (Reactions 1–3). Neither enzymatic inhibition nor Compound III formation (an inactive enzyme intermediate) has been observed in the thiol concentration range investigated (10–500 μM).

As Table 2 shows, three groups of thiols have to be distinguished relating to their demand for exogenously added hydrogen peroxide for reaction: (i) Aromatic thiols have been shown to be good electron donors for both enzyme intermediates, but their oxidation is strictly dependent on hydrogen peroxide addition as the steady-state data for 2-mercaptopurine in Figs. 2a and 3a show. By addition of 10 μM H_2O_2 both thiol oxidation rate and oxygen consumption rate are increased by a factor of 15 (a similar acceleration has been observed with thiophenol). Without exogenously added peroxide the reaction terminates very fast. This is in agreement with the observation that without H_2O_2 ferriperoxidase is the dominant form of the enzyme in steady-state. Thus the regeneration of hydrogen peroxide and consequently the turnover of ferriperoxidase represents the rate-liming step in the oxidation of aromatic thiols. Upon addition of H_2O_2 Compound II accumulates. These facts indicate that arylthiyl radicals do not seem to follow Reactions 4–8, which have been established for aliphatic thiols. Though being effectively generated by HRP, arylthiyl radicals do not have the capabilities for sufficient regeneration of hydrogen peroxide via free radical chain reactions. This confirms previous observations that in aqueous systems arylthiyl radicals have properties dissimilar to those of thiyl radicals from aliphatic –SH functions [19].

With regard to aliphatic thiols, there are two more groups to be distinguished concerning the demand for exogenously added hydrogen peroxide (Table 2), namely (ii) those thiols whose steady-state behaviour (both thiol oxidation and oxygen consumption) has been influenced by addition of H_2O_2 (*N*-acetylcysteine, glutathione, homocysteine), and (iii) those thiols whose oxidation rate and oxygen consumption was more or less independent of it (cysteine, cysteamine, cysteine methyl ester, cysteine ethyl ester, 2,3-dimercaptopropanol) as long as aerobic conditions are considered. The former group (ii) contains thiols which have been shown to be extremely bad electron donors for both enzyme intermediates (with re-

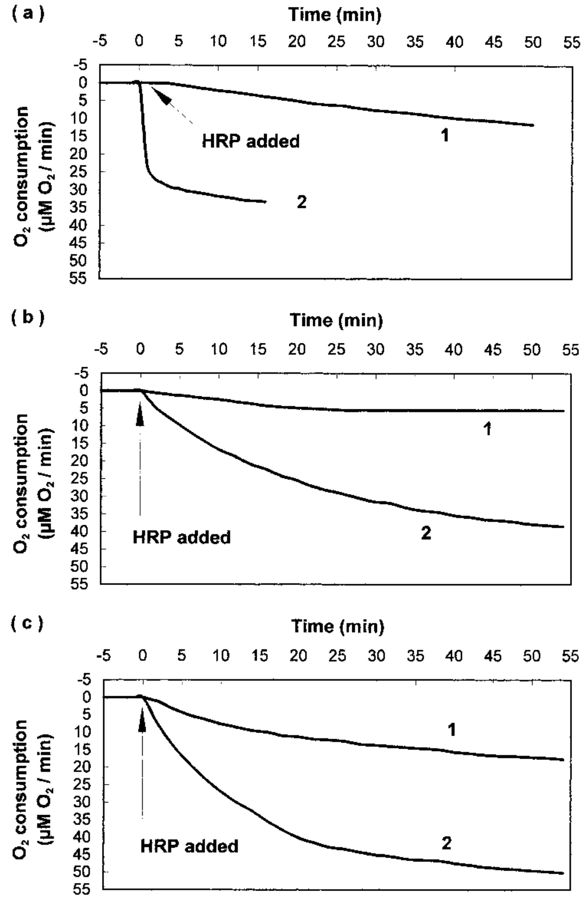

Fig. 2. Time courses of oxygen consumption during HRP (1.7 μM) mediated thiol (500 μM) oxidation in the absence (1) and presence (2) of hydrogen peroxide (10 μM). Incubation mixtures contained thiol [2-mercaptopurine (a), glutathione (b), cysteine (c)], 1 mM EDTA and 50 mM phosphate buffer, pH 7.0. Temperature was set to 30°C and reaction was started by addition of enzyme.

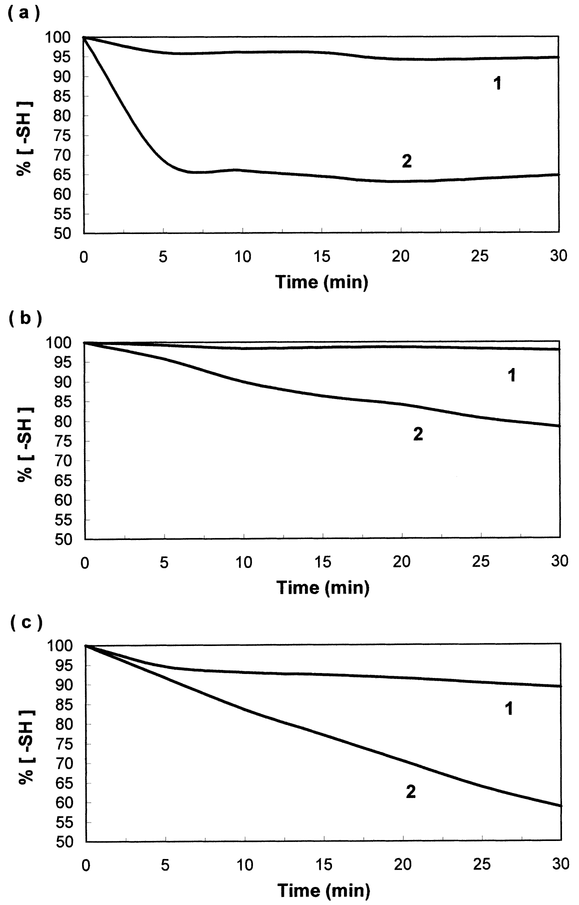

Fig. 3. Time courses of thiol (500 μM) oxidation mediated by HRP (1.7 μM) in the absence (1) and presence (2) of hydrogen peroxide (10 μM). Incubation mixtures contained thiol [2-mercaptopurine (a), glutathione (b), cysteine (c)], 1 mM EDTA and 50 mM phosphate buffer, pH 7.0. Temperature was set to 30°C and reaction was started by addition of enzyme.

action rates with Compound II smaller than 10 M^{-1} s^{-1}). The second group showed rates for k_3 between 11 and 1600 M^{-1} s^{-1}. These rather small values for k_3 lead to Compound II accumulation during turnover, indicating that reduction of Compound II back to ferriperoxidase is the rate-limiting step [12] and not regeneration of hydrogen peroxide via non-enzymatic free-radical chain reactions. This is underlined by the fact that addition of hydrogen peroxide to the reaction medium had only a small effect on overall thiol oxidation. With 10 μM hydrogen peroxide (Table 2) the effect on thiol oxidation was more pronounced with decreasing values for k_2 and k_3 and increasing values for $pK_{a,SH}$. Initial thiol oxidation rates of *N*-acetylcysteine, glutathione (Fig. 3b) and homocysteine (group (ii)) have been shown to be increased by factors of 10, 7.5 and 3, whereas oxygen consumption has been increased by factors of 75, 4 (Fig. 2b), and 2, respectively. On the contrary, cysteine oxidation (Fig. 3c) was increased by 65% and oxygen consumption rate by 330% (Fig. 2c), but with cysteamine and all other thiols of group (iii) addition of hydrogen peroxide showed no impact on either thiol oxidation or oxygen consumption rates.

As long as thiols with similar dissociation constants for the mercapto group are compared ($pK_{a,SH} = 8.3–8.9$), overall thiol oxidation and oxygen consumption reflect very well the rate constants measured by transient-state kinetics, with the activity of cysteamine > cysteine > homocysteine > glutathione. The higher the rates of these thiols with Compound I and II are (Table 1), the higher are both thiol oxidation and oxygen consumption rates (Table 2). Oxygen consumption reflects non-enzymatic free radical reactions by which hydrogen peroxide is regenerated with the consequence that at higher oxygen consumption rates the system becomes independent of added peroxide.

From all thiols investigated oxidation of *N*-acetylcysteine by HRP showed the lowest oxidation rate. Though having a similar reactivity to glutathione with HRP Compound I and II, in the absence of hydrogen peroxide no thiol oxidation and oxygen consumption are observed. Upon addition of H_2O_2 the initial oxidation rate is increased dramatically, but the reaction terminates very fast. On the other hand, penicillamine—though being the worst electron donor for Compound I and Compound II—thiol oxidation (and oxygen consumption) is 3 times (5 times) the rate observed with cysteine. These facts demonstrate very well the important role of the deprotonated form of aliphatic thiols in reaction with thiyl radicals produced by HRP. Thiyl radicals react 10^4 to 10^5 times faster with thiolates than with thiols [7] producing the disulphide radical anion, which above all is responsible for oxygen reduction and consequently hydrogen peroxide regeneration (Reactions 4–8). At pH 7.0 the percentage of the thiolate form of penicillamine is about 11%, whereas with *N*-acetylcysteine it goes to zero (Table 1). So the present in-vitro study unequivocally shows that—though being generated at similar rates—penicillamine has oxidative behaviour (in the sense that it generates hydrogen peroxide, which is permanently consumed by the enzyme) and *N*-acetylcysteine antioxidative behaviour. Glutathione, the biologically very important tripeptide which protects cells against oxidation [20], behaves similarly to *N*-acetylcysteine. Its oxidation by HRP is extremely slow as long as hydrogen peroxide derives only from free-radical chain reactions (Figs. 2b and 3b), again reflecting the low percentage of the deprotonated form at physiological pH.

By some means the two esters of cysteine are special cases. These substances are oxidized very effectively by HRP. In addition, at physiological pH the thiolate form predominates with the consequence that addition of hydrogen peroxide had nearly no effect on overall thiol oxidation, indicating that its regeneration was very effective. This underlines the very pro-oxidative nature of cysteine esters in this definite in-vitro system. On the other hand, oxidation of α-lipoic acid and oxygen consumption in the absence of added hydrogen peroxide (though showing about 10 times the reactivity of penicillamine with both peroxidase intermediates) has been extremely slow, because at pH 7 the deprotonated form does not exist.

Summing up, low-molecular-weight thiols play an important role in biological systems [20] and in many reactions the one-electron oxidation products (thiyl radicals) are involved. With HRP as radical generator an enzymatic system exists capable of oxidizing a wide variety of thiols to their corresponding thiyl radicals, though the effectivity of these reactions varies dramatically and is a consequence of the thiol structure. The transient-state data give us, for the first time, real generation rates at the enzyme and therefore a better basis for further investigation and discussion of the behaviour of thiyl radicals in an aqueous reaction mixture, which is a consequence of their generation rates, the percentage of thiolate and their aliphatic or aromatic nature.

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