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MECHANISTIC ASPECTS OF THE OXIDATION OF PHENOTHIAZINE DERIVATIVES BY METHEMOGLOBIN IN THE PRESENCE OF HYDROGEN PEROXIDE

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Abstract—Mechanistic aspects of the reaction of hydrogen peroxide with methemoglobin with respect to phenothiazine oxidation have been studied. Three phenothiazines, methoxy- (MoPZ), chlor- (CPZ) and methoxycarbonylpromazine (MaPZ), have been used. These phenothiazines differ only in substitution at the 2-position, which contributes substantially to the electron-donating properties of these compounds. Reaction with hydrogen peroxide oxidizes methemoglobin to ferrylhemoglobin, which contains iron(IV)-oxo porphyrin moiety and a protein radical. The phenothiazines are oxidized by ferrylhemoglobin in the presence of H_2O_2 mainly to their sulfoxides, with a radical cation as intermediate. The conversion rates (MoPZ > CPZ > MaPZ) decrease with the electron-withdrawing ability of the 2-substituent, as indicated by Hammett σ_{para} values. Hydrogen peroxide consumption during the reaction is similar for the three phenothiazines. Denaturation reactions that occur upon exposure of methemoglobin to hydrogen peroxide have been investigated. For this heme–protein cross-linking was studied by means of heme retention by the protein after methyl ethyl ketone extraction. Furthermore, oxygen consumption during the reaction was assayed, which indicates formation of protein–peroxy radicals. The extent of both heme–protein cross-linking and oxygen consumption is decreased by phenothiazines in the same order as the phenothiazine conversion rate. CPZ sulfoxide is not converted by methemoglobin in the presence of hydrogen peroxide, and CPZ sulfoxide shows no effect on heme–protein cross-linking and oxygen consumption. The results are explained by electron transfer from phenothiazine to the protein radical. Stronger electron donors (MoPZ > CPZ > MaPZ) are converted faster and by reducing the protein radical they better protect hemoglobin against denaturation. A catalytic cycle, that takes into account our observation and the existing knowledge of hemoglobin oxidation states, is presented.

The phenothiazine drugs (PHs‡) exist as a wide range of structurally related compounds which vary in substitution at the 2- and 10-position, and which are mainly used as neuroleptics and antihistamines [1]. PHs have excellent electron-donating properties [2] leading to the formation of relatively stable radical cations ($PH^{+\bullet}$) [3]. These $PH^{+\bullet}$ are generally assumed to be involved as intermediates in the oxidation of PHs to sulfoxides (PHSO) and ring-hydroxylated products *in vivo* [4]. Recently we reported the formation of chlorpromazine radical cation ($CPZ^{+\bullet}$) during the reaction of chlorpromazine (CPZ) with methemoglobin (metHb) in the presence of H_2O_2 [5].

Reaction with H_2O_2 oxidizes metHb to ferrylhemoglobin (ferrylHb). FerrylHb has an iron(IV)-oxo porphyrin moiety (ferryl heme, represented as $Fe^{IV} = O$), comparable with horseradish peroxidase (HRP) compound II [6]. Upon reaction with H_2O_2 one oxidation equivalent is retained on the apoprotein leading to the formation of transient protein radicals [7, 8]. The identity of the amino acid residue that carries the unpaired electron density has not been unambiguously determined, but there are indications that a tyrosine residue in the vicinity of the heme group is the locus [8]. The protein radical decays much faster than the ferryl heme moiety [8]. Thereafter, a more stable ferrylHb species without protein radical remains [9], which is spectrophotometrically identical to ferrylHb with the protein radical. Decay of the protein radical has been implicated in formation of covalently cross-linked dimers in sperm whale myoglobin [10, 11] and human Hb [10] and in formation of heme–protein cross-links in horse myoglobin [12]. We have demonstrated that in the reaction of metHb with H_2O_2 also heme–protein cross-linking occurs [5]. Addition of molecular oxygen to the protein radical has been proposed as another reaction involving the protein radical [8, 13]. This leads to formation of a protein–peroxy radical. The peroxy radical has been implicated in the epoxidation of olefins by metmyoglobin as well as metHb in the presence of H_2O_2 [13, 14].

In the present study several aspects of the reaction of three PHs with metHb in the presence of H_2O_2 have been investigated. These PHs, MoPZ, CPZ and MaPZ, differ only in their 2-substituent (Table

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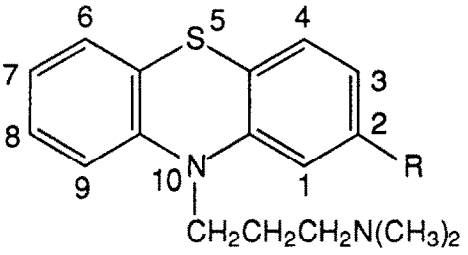
‡ Abbreviations: PH, PHSO, $PH^{+\bullet}$, phenothiazine (in general), its sulfoxide and its radical cation, respectively; CPZ, MoPZ, MaPZ; chlor-, methoxy-, methoxycarbonylpromazine, respectively; CPZSO, MoPZSO, MaPZSO, their sulfoxides, respectively; $CPZ^{+\bullet}$, $MoPZ^{+\bullet}$, $MaPZ^{+\bullet}$, their radical cations, respectively; Hb, hemoglobin (in general); metHb, ferrylHb, met-, and ferrylhemoglobin, respectively; HRP, horseradish peroxidase; MEK, methyl ethyl ketone.

Table 1. Structures of phenothiazine derivatives and Hammett σ_{para} values of 2-substituent



Compound		R	σ_{para}*
Methopromazine	(MoPZ)	OCH_3	−0.27
Chlorpromazine	(CPZ)	Cl	0.23
Methoxycarbonylpromazine	(MaPZ)	$COOCH_3$	0.45

* From Ref. 15.

1). These substituents differ substantially in electronic properties, as indicated by their Hammett σ_{para} values [15]. Therefore, if electron transfer from PH to the Hb protein radical is involved, the reaction should reflect the electron-donating property of the 2-substituent. We investigated the conversion rates of MoPZ, CPZ and MaPZ by metHb plus H_2O_2 together with the effects on H_2O_2 and oxygen consumption, and on heme–protein cross-linking. We report that PHs protect against oxygen consumption and formation of heme–protein cross-links induced by the reaction of metHb with H_2O_2. The extent of the protection is dependent on the electron donating property of the 2-substituent.

MATERIALS AND METHODS

MaPZ maleate, MoPZ maleate and CPZSO were gifts from Rhône-Poulenc (Paris, France). [6,7,8,9-^{3}H]CPZ hydrochloride (sp. act. 23.3 Ci/mmol) was from New England Nuclear (Dreieich, F.R.G.). CPZ hydrochloride, HRP (donor: H_2O_2 oxidoreductase; EC 1.11.1.7; activity as defined by Sigma), and catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) Buffers were made of $Na_2HPO_4.2H_2O$ and KH_2PO_4 (Fluka AG, Buchs, Switzerland) and always had a pH of 6.5 and an ionic strength of 0.1 M. All other chemicals were obtained from E. Merck (Darmstadt, F.R.G.). Solutions were prepared with demineralized water purified through a Millipore purification system (15 MΩ water). Oxyhemoglobin was isolated from human whole blood according to the method of Eyer *et al.* [16] and contained no superoxide dismutase, no catalase and no glutathione peroxidase activity, as described previously [17]. MetHb was prepared from this oxyhemoglobin stock solution (stored at −80°) by the addition of $K_3Fe(CN)_6$, and subsequent elution over Sephadex G-25 M with buffer. MetHb solutions were quantitated spectrophotometrically using a molar absorptivity at 522 nm of $3.0 \times 10^4\ M^{-1}\ cm^{-1}$ for the tetrameric molecule [18].

Conversions of phenothiazine derivatives by methemoglobin in the presence of hydrogen peroxide. Typical reaction mixtures contained 10 μM metHb, 50 μM PH and 100 μM H_2O_2 in 3.0 mL air-saturated phosphate buffer, pH 6.5. Reactions were performed in a cuvet, thermostatted at 30° and directly monitored spectrophotometrically (Perkin–Elmer Lambda 5 UV/VIS). The reactions were started by the addition of H_2O_2. Where indicated other compounds were added or other conditions used. In the case of CPZ, a second reaction cycle was performed by adding CPZ after 15 min and re-starting the reaction by adding H_2O_2. This reaction was also performed by [^{3}H]CPZ (sp. act. 25 mCi/mmol), as reported previously [5], to investigate irreversible binding to metHb. Samples of 50 μL were taken, diluted into 150 μL acetonitrile and mixed on a Vortex mixer. This procedure stops the reaction by precipitation of metHb. After centrifugation of 15,000 rpm for 5 min the supernatant was analysed by HPLC. MoPZ, MaPZ, CPZ and their sulfoxides were recovered for over 99% from metHb-containing solutions, using this procedure.

Oxygen and hydrogen peroxide consumption. The above-mentioned reactions were also performed in a closed glass vessel (1.7 mL) thermostated at 30° and equipped with a stirrer and an oxygen electrode (Clark type; connected to a YSI Model 53 Biological oxygen monitor). Reactions were started by addition of H_2O_2 or PH (both preequilibrated at 30°) through a capillary aperture, and changes in oxygen tension were recorded. H_2O_2 consumption during these reactions was determined with the same apparatus. In that case, catalase (final concn 20 μg/mL) was added at timed intervals after the initiation of the reaction. The increase of the oxygen concentration then observed is a measure for the H_2O_2 still present at that time. A calibration curve was used to determine the concentration of H_2O_2.

Heme destruction and heme–protein cross-linking. After completion of the various reactions, an extra 50 μM PH was added to accelerate decay of remaining

ferrylHb [5]. The absorbance of the Soret band at 405 nm of the 10-fold diluted reaction mixture was determined. Decrease of absorbance at 405 nm is due to modifications in the heme and is considered to be a measure for heme destruction. The extent of heme destruction was calculated relative to intact metHb, for which a molar absorptivity at 405 nm of $6.72 \times 10^5\ M^{-1}\ cm^{-1}$ was used [5].

After completion of the reactions, the mixture was also extracted with an equal volume of methyl ethyl ketone (MEK) at pH 2.5 and 4°, to remove the heme moiety from the holoprotein [19]. The organic layer was separated from the aqueous phase. The aqueous phase was dialysed exhaustively against phosphate buffer, pH 6.5, to remove MEK. The relative non-extractable heme content was calculated using the molar absorptivity at 405 nm of intact metHb. Alternatively, the loss of heme was also calculated based on the absorbance of metHb after reaction, in this way accounting for changes in Soret absorption due to heme destruction.

HPLC analysis. For HPLC analyses a system was built from a Model 9000-4002 (Kratos) solvent delivery system combined with a SP 8775 (Spectra Physics) autosampler and a Model 757 (Kratos) absorbance detector, set at 248 nm. Samples of 50 μL were chromatographed on a 5 μm Spherisorb Cyano-column (15 cm × 4.6 mm i.d.; Phase Separation Ltd), thermostated at 25°. The mobile phase consisted of a mixture of 80% (w/w) methanol and 20% (w/w) 0.06 M ammonium acetate, pH 6.5. The flow rate was 1.5 mL/min.

RESULTS

Several aspects of the reaction of PH derivatives with metHb plus H_2O_2 have been analysed: (1) rate of PH conversion, (2) spectral changes, (3) rate of H_2O_2 consumption, (4) rate of oxygen consumption, whether or not in the presence of PH during the reaction, and (5) heme destruction and heme–protein cross-linking after the reaction was completed.

Rate of phenothiazine drug conversion

The conversion of MoPZ, CPZ and MaPZ by metHb plus H_2O_2 is presented in Fig. 1. The conversion rate decreased in the order MoPZ > CPZ > MaPZ and shows an inverse relation with the electron-withdrawing ability of the 2-substituent, as indicated by the Hammett σ_{para} value (Table 1). MoPZ was transformed completely within 3.2 min. The conversion of CPZ and MaPZ stopped after 6–7 min, with 3.5 and 14.8 μM of the parent compounds remaining, respectively. After this time all H_2O_2 appeared to be utilized (see below). The main product was PH sulfoxide, as determined by HPLC analysis and from the absorption spectra of the relevant HPLC peaks. We reported previously that conversion of CPZ yields approximately 60% CPZSO and that the remaining 40% is covalently bound to apohemoglobin [5]. For MoPZ and MaPZ the yield of sulfoxide has been estimated using sulfoxide standard solutions obtained from conversion of MoPZ and MaPZ by HRP (40 μg/mL) and excess H_2O_2. Under these conditions sulfoxide is the only product observed. The yields of

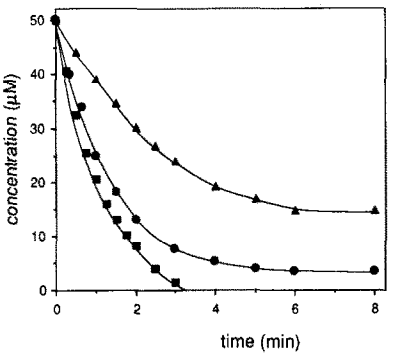


Fig. 1. Time course of the conversion of PHs by metHb in the presence of H_2O_2. PH (50 μM) was incubated with metHb (10 μM) in phosphate buffer, pH 6.5 (ionic strength 0.1 M) at 30°, and reaction was started by adding H_2O_2 (100 μM). The indicated points are the mean of four determinations with SEM generally <0.5 μM. MoPZ (■), CPZ (●), MaPZ (▲).

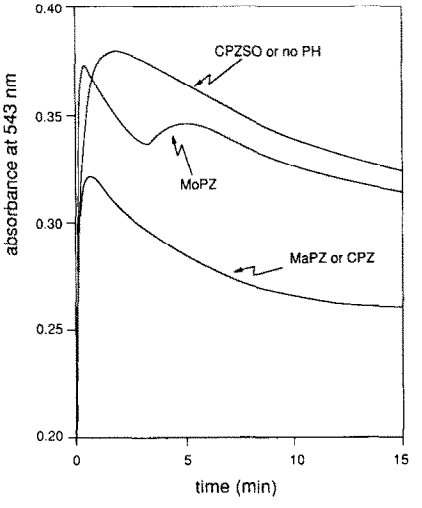


Fig. 2. Changes of absorbance at 453 nm during reaction of metHb with H_2O_2 in the presence of MoPZ, CPZ, MaPZ or CPZSO. For conditions see the legend to Fig. 1. CPZ and MaPZ provoked identical absorbance changes. CPZSO showed the same absorbance changes as metHb plus H_2O_2 in the absence of PH.

MoPZ sulfoxide (MoPZSO) and MaPZ sulfoxide (MaPZSO) in the Hb/H_2O_2 reaction were approximately 95 and 50%, respectively.

Control experiments showed that MoPZ, CPZ and MaPZ were not transformed by either metHb or H_2O_2 alone. The conversion of CPZ was not influenced by maleate (50 μM), which is the counter ion of MoPZ and MaPZ.

Spectrophotometric analyses

MetHb is oxidized by H_2O_2 to ferrylHb which has a characteristic absorption spectrum with a maximum of 543 nm. The time course of absorbance at 543 nm during the reactions is shown in Fig. 2. The ferrylHb

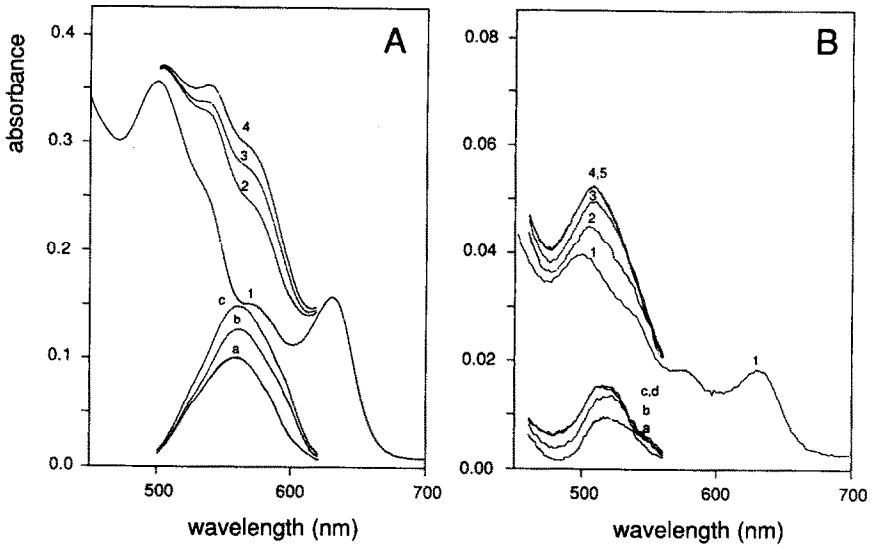


Fig. 3. Radical cation formation during the reaction of MoPZ (A) or MaPZ (B) with metHb and H_2O_2. (A) MoPZ (0.5 mM) was incubated as described in the legend to Fig. 1. (B) MaPZ (0.5 mM) was incubated with 1 μM metHb and 1.0 mM H_2O_2 under further identical conditions as in the legend to Fig. 1. Spectra were recorded at intervals (in sec): 0 (1), 15 (2), 30 (3), 45 (4) and 60 (5). Difference spectra were constructed by substracting spectrum 1 from the other spectra: 2 – 1 (a), 3 – 1 (b), 4 – 1 (c), 5 – 1 (d).

concentration remained lower in the presence of CPZ and MaPZ than in the reaction without PH. In the presence of MoPZ a higher absorbance than in the absence of PH was observed during the first min, which was due to absorbance by the radical cation of MoPZ ($MoPZ^{+\bullet}$). $MoPZ^{+\bullet}$ has an absorption maximum at 562 nm [3] and was produced in such a high concentration that it was easily detected by recording difference spectra (Fig. 3). The high $MoPZ^{+\bullet}$ concentration is not only due to a faster rate of formation but also to a slower decay of $MoPZ^{+\bullet}$ than the other PH radicals [3]. The minimum observed at 3.2 min in the time course curve for MoPZ corresponded to the time interval in which MoPZ was transformed completely. The remaining H_2O_2 was used to reproduce ferrylHb leading to a second maximum after 5 min. The occurrence of the MaPZ radical ($MaPZ^{+\bullet}$) in the reaction could spectrophotometrically only be demonstrated by changing the concentrations of the reactants, i.e. 0.5 mM MaPZ, 1 μM metHb and 1.0 mM H_2O_2 (Fig. 3), as we reported previously for $CPZ^{+\bullet}$ [5]. $MaPZ^{+\bullet}$ has an absorption maximum at 514 nm [3].

Decay of ferrylHb was accelerated by PH derivatives, whether or not H_2O_2 was actually present, as we reported previously for CPZ [5]. MoPZ and MaPZ showed the same influence on the ferrylHb decay rates as CPZ. This accelerating effect of PHs on ferrylHb decay only resulted in significant PH transformation if H_2O_2 was present.

Hydrogen peroxide and oxygen consumption

The time course of H_2O_2 concentration present during the reactions of PHs with metHb plus H_2O_2 has been assayed and is presented in Fig. 4. The decrease of H_2O_2 concentration was nearly identical in the presence of the three PHs. Only in the presence of MoPZ the H_2O_2 consumption was somewhat retarded after 3 min. This can be explained by the fact that MoPZ has been converted completely after 3.2 min. In the presence of CPZSO the rate of H_2O_2 consumption was the same as in the absence of PH and was somewhat slower than in the presence of PH (Fig. 4).

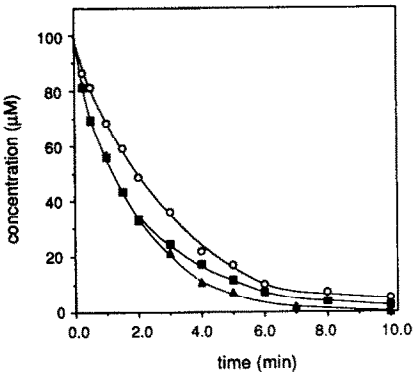


Fig. 4. Decrease of H_2O_2 concentration in the reaction with metHb in the presence of MoPZ, MaPZ or CPZSO. For conditions see the legend to Fig. 1. The indicated points are the mean of three assays with SEM generally <1 μM. MoPZ (■), MaPZ (▲), CPZSO (○). H_2O_2 consumption in the absence of PH was the same as in the presence of CPZSO, and CPZ showed the same H_2O_2 consumption as MaPZ.

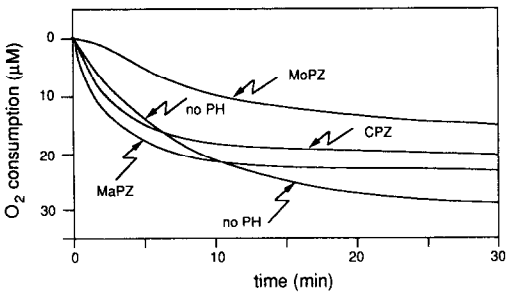


Fig. 5. Oxygen consumption during reaction of metHb with H_2O_2 in the absence and presence of MoPZ, CPZ or MaPZ. For conditions see the legend to Fig. 1. In the presence of CPZSO the oxygen consumption was the same as in the absence of PH.

The time course of oxygen consumption during the reaction of metHb with H_2O_2 is shown in Fig. 5. In the reaction of 10 μM metHb with 100 μM H_2O_2, approximately 30 μM oxygen was consumed in the first 30 min. Oxygen consumption continued after all H_2O_2 had been utilized. The fact that oxygen consumption does not parallel H_2O_2 consumption indicates that these processes are not directly related to one another. The effect of PH derivatives on the initial rate of oxygen consumption (MoPZ < CPZ < MaPZ) was reciprocal with the rate of PH conversion (Fig. 5). The total oxygen consumption after 30 min showed the same order. The presence of CPZSO had no effect: the oxygen consumption was identical to that in the absence of PH.

In the presence of 1 mM sodium azide, which binds tightly to the heme group, the characteristic absorption spectrum of metHb–azide complex did not change upon H_2O_2 addition, indicating that ferrylHb was not formed. Under this condition no oxygen consumption occurred and PHs were not oxidized. H_2O_2 consumption could not be determined, because azide inhibits catalase.

As a control, spontaneous oxygen evolution from 100 μM H_2O_2 was observed to be less than 0.5 μM during a 15 min interval.

Heme destruction and heme–protein cross-linking

The absorbance of the Soret band at 405 nm was decreased after reaction of metHb with H_2O_2 (Table 2). The loss of Soret absorbance, due to heme destruction, was partly prevented by PHs, dependent on their concentrations (Table 2). The difference between the effects of various PHs was not large. The order of loss of Soret absorbance was MaPZ > CPZ > MoPZ. CPZSO did not prevent loss of Soret absorbance. The covalent binding between the heme moiety and the apoprotein was studied with MEK extraction [5, 12]. The results are also included in Table 2. It is not known whether the loss of heme absorbance at 405 nm is caused by a loss of heme from the protein or by a change in the absorption due to heme destruction. Therefore, the heme retention upon MEK extraction has been calculated relative to both the absorbance of intact metHb (I) and the absorbance observed after completion of the reaction (II) (Table 2). Both methods yield similar results: the extent of heme–protein cross-links was reduced when PHs were included in the reaction of metHb with H_2O_2, in the order MoPZ > CPZ > MaPZ. CPZSO gave no reduction of the heme–protein cross-linking.

Second reaction cycle

The reaction with CPZ was executed a second

Table 2. Effect of PHs on Soret absorbance and on non-extractable heme content after reaction of metHb with H_2O_2*

Additives	Loss A_{Soret}† (%)	Non-extractable heme‡ I (%)	II (%)
Control, no PH, no H_2O_2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
no PH	18.6 ± 1.3	10.6 ± 0.9	13.0 ± 0.9
MoPZ 50 μM	8.1 ± 0.7	—	—
100 μM	5.2 ± 0.7	3.1 ± 0.3	3.3 ± 0.3
CPZ 50 μM	10.3 ± 0.9	5.3 ± 0.6	6.0 ± 0.7
100 μM	6.6 ± 0.8	4.2 ± 0.3	4.4 ± 0.4
MaPZ 50 μM	11.9 ± 0.9	—	—
100 μM	8.8 ± 0.9	5.1 ± 0.4	5.5 ± 0.5
CPZSO 100 μM	19.0 ± 1.3	10.8 ± 0.9	13.3 ± 1.0

Values are the mean ± SEM of three determinations.

* Conditions as described in the legend to Fig. 1. For determinations see Materials and Methods.

† Calculated relative to absorbance of metHb at 405 nm (molar absorptivity at 405 nm of $6.72 \times 10^5\ M^{-1}\,cm^{-1}$).

‡ I: Calculated relative to A_{Soret} of metHb. II: Calculated relative to A_{Soret} after reaction.

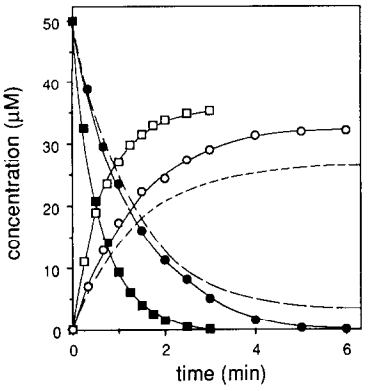


Fig. 6. Conversion of CPZ into CPZSO by metHb plus H_2O_2 during the second execution of the reaction. For conditions see the legend to Fig. 1. After 15 min the reaction was repeated in the same mixture by adding again CPZ up to 50 and 100 μM H_2O_2. This second reaction was performed after CPZ was either absent (●) or present (■) during the first reaction performance. CPZ, closed symbols; CPZSO, open symbols. Broken lines represent the conversion of CPZ (———) into CPZSO (----) during the first reaction [5]. The indicated points are the mean of four assays with SEM generally <0.5 μM.

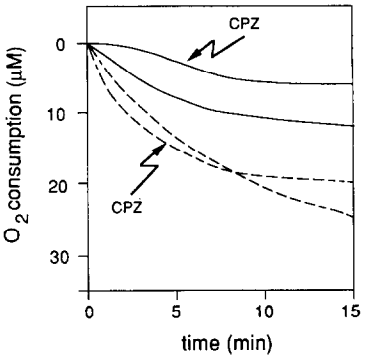


Fig. 7. Oxygen consumption during reaction of metHb with H_2O_2 in the absence and presence of CPZ during both the first and second reaction. The first reaction was performed as in Fig. 1. The second reaction was started 15 min later in the same mixture by adding again 50 μM CPZ and 100 μM H_2O_2. First reaction (----), second reaction (———). The presence of CPZ is indicated.

time in the same reaction mixture after consumption of all H_2O_2 and nearly all CPZ, by adding 46.5 μM CPZ and 100 μM H_2O_2 after 15 min. The results are shown in Fig. 6. The rate of CPZ conversion was increased in the second cycle. The conversion of CPZ was not influenced by CPZSO, indicating that the faster conversion in the second reaction was not due to the presence of CPZSO. In the first reaction, conversion of CPZ yields 60% CPZSO and 40% covalently bound CPZ throughout the reaction [5]. In the second reaction CPZSO yield was not a fixed fraction of the converted amount of CPZ throughout the reaction, but this CPZSO fraction increased from 63% at the start to about 90% at the end of the conversion (Fig. 6). The fraction of [^{3}H]CPZ that was bound covalently to apohemoglobin during the reaction decreased accordingly (results not shown). The mass balance of CPZ, CPZSO and covalently bound CPZ was 99 ± 4% of the initial level throughout the reaction.

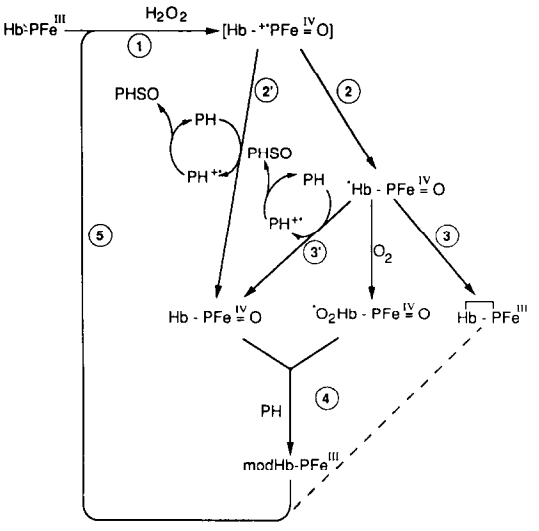
In the second reaction, the H_2O_2 consumption was the same as in the first reaction. The oxygen consumption, however, differed from the first reaction: showing no decrease of oxygen concentration during the first 3 min after the re-start and a small decrease thereafter (Fig. 7). When only H_2O_2 and no CPZ had been added in the second reaction, oxygen consumption also showed a rate that was considerably smaller than during the first reaction (Fig. 7). If both CPZ and H_2O_2 were added in the second reaction the oxygen consumption was even further decreased.

If the first reaction was performed with H_2O_2 alone and CPZ was omitted, CPZ conversion in the second reaction was slower that when CPZ had been included in the first reaction (Fig. 6). The conversion now was somewhat faster than CPZ conversion in the first reaction. When CPZ was converted completely in the second reaction this yielded approximately 64% CPZSO and 34% covalent bound CPZ.

DISCUSSION

Upon reaction with H_2O_2, metHb is transformed into ferrylHb. In the presence of H_2O_2, ferrylHb oxidizes MoPZ, CPZ and MaPZ at different reaction rates (Fig. 1) and their sulfoxides are the main products. The PH conversion rates decrease with the electron-withdrawing ability of the 2-substituent, as indicated by Hammett σ_{para} values (Table 1). This substituent is para to the sulfur atom which is the reaction center [4]. Together with the observation of $PH^{+\bullet}$ as an intermediate (Fig. 3), these results are in agreement with electron transfer from PH to ferrylHb. PH radicals are further transformed by non-enzymatic reactions. A half-regeneration reaction produces parent compound and sulfoxide with yields of 50% of each product [20]. Previously we reported that CPZ irreversibly binds to the apoprotein [5]. This irreversible binding also results from a non-enzymatic reaction of $CPZ^{+\bullet}$. $MoPZ^{+\bullet}$ and $MaPZ^{+\bullet}$ were also observed during the reaction of MoPZ and MaPZ with metHb in the presence of H_2O_2. MoPZ is almost completely converted into MoPZSO, indicating that irreversible binding of MoPZ to the protein is very small or absent. MaPZ is converted into MaPZSO in approximately 50% yield and no other substantial products are observed by HPLC analysis. Therefore, irreversible binding of MaPZ to metHb is likely.

H_2O_2 is consumed during the reaction of PH with metHb in the presence of H_2O_2. The H_2O_2 consumption is similar whether MoPZ, MaPZ or CPZ reacts with metHb plus H_2O_2. This indicates that no direct relation between the rate of PH conversion and H_2O_2 consumptions exists. H_2O_2 consumption seems to be influenced primarily by reactions involving Hb itself. This is deduced from



Scheme 1. Proposed catalytic cycle of the reaction of metHb with H_2O_2 and PH as substrate. PH is oxidized either in step 2′ or step 3′ (or both). Structures are described in the text.

the fact that in the absence of PH also H_2O_2 is consumed by metHb, and that this consumption is not much increased in the presence of a PH derivative (Fig. 4). H_2O_2 is used to produce ferrylHb. The protein radical in ferrylHb decays fast and its decay ultimately results in formation of covalently cross-linked dimers [10, 11] and heme–protein cross-links [12].

In the reaction of metHb with H_2O_2, oxygen is also consumed (Fig. 5). This oxygen consumption is blocked by azide. Azide binds tightly to the heme and thereby prevents formation of ferrylHb. This indicates that consumption of oxygen is related to formation of ferrylHb. The oxygen consumption is most probably caused by oxygen addition to the protein radical of ferrylHb, leading to the formation of protein–peroxy radicals, which ultimately modify the apoprotein [8, 13]. After all H_2O_2 has been utilized, consumption of oxygen continues, indicating that the protein radical of ferrylHb is relatively slowly oxidized or that decay of the ferryl moiety also provokes oxygen consumption. The H_2O_2 consumption in the absence of PHs is used in reactions leading to denaturation of Hb. In the presence of PH a part of the oxidizing equivalents originating from H_2O_2, is used to oxidize PH.

Based on the reactions described above, a reaction sequence can be postulated in which metHb participates in a catalytic cycle. In this cycle ferrylHb is produced, which abstracts electrons from a substrate and ferrylHb is reduced to the resting (modified) metHb. A catalytic cycle is presented in Scheme 1. This scheme takes into account all our observations concerning the reaction of PHs with metHb in the presence of H_2O_2 and the existing knowledge of Hb oxidation states.

In this scheme, ferrylHb with a protein radical ($^{\bullet}$Hb-PFe^{IV} = O) is formed in two steps. In step 1, metHb (Hb-PFe^{III}) is oxidized by H_2O_2 to a peroxidase compound I-like species ([Hb-$^{+\bullet}$PFe^{IV} = O]). This species contains an iron(IV)-oxo group and a protoporphyrin π radical cation ($^{+\bullet}$P). The existence of this intermediate has never been established but has been postulated as an intermediate in the formation of ferrylHb [7, 21]. The short life-time of the compound I analogue might be due to localization of an endogenic amino acid residue near the porphyrin moiety. In step 2, this amino acid residue is easily oxidized, transferring an electron to the porphyrin π radical cation [8, 21]. The protein radical in ferrylHb is probably located on a tyrosine residue [8, 11]: in the case of equine metHb Tyr 42 has been reported [13]. The tyrosine radical is probably not only in contact with the heme pocket but also with the medium [13] and is therefore accessible to the bulky PH derivatives. The protein radical is involved in reactions that modify metHb. In step 3 denaturation reactions observed in this study are indicated: heme–protein cross-links (Hb-PFe^{III}) and reaction with oxygen yielding protein–peroxy radicals ($^{\bullet}$O$_2$Hb-PFe^{IV} = O).

PHs are able to donate an electron to the protein radical (step 3′). The protein radical is reduced and rendered harmless with respect to denaturation reactions. In this reaction a stable ferrylHb (Hb-PFe^{IV} = O) is formed. This is in agreement with the observed protective effect of PHs against the H_2O_2-induced heme–protein cross-links. This effect decreases in the order MoPZ > CPZ > MaPZ (Table 2). This suggests that better electron donating PHs, like MoPZ, will reduce the protein radical more easily and thereby more efficiently prevent reactions that result in heme–protein cross-links than PHs with smaller electron-donating ability, e.g. MaPZ. The decreasing effect of PHs on oxygen consumption, which is of the same order as above, can be explained by prevention of the formation of the protein–peroxy radicals by electron transfer of PH to the protein radical as well. In fact, the reaction of PH with the protein radical competes with reactions leading to Hb denaturation. As a consequence of the electron transfer, PH is oxidized to its radical cation. PH$^{+\bullet}$ either binds irreversible to the apoprotein [5] or reacts further to yield 50% parent compound and 50% sulfoxide [20]. An alternative mechanism for PH oxidation and formation of ferrylHb without protein radical is illustrated by step 2′. In this step, PH donates its electron directly to the compound I-like intermediate. In fact, this represents a competition between PH and an endogenic amino acid residue for electron transfer to the porphyrin radical. Upon transfer of an electron to the porphyrin radical, PH prevents formation of the protein radical and thereby also formation of the protein–peroxy radical and heme–protein cross-links. Our results do not permit to discriminate between both mechanisms. However, the very short life-time of the compound I analogue and the better accessibility of the protein radical [13] for electron transfer from PHs favour the apoprotein as a site for electron transfer.

In step 4 the ferryl moiety in both stable ferrylHb (Hb-PFe^{IV} = O) and ferrylHb with a protein peroxyradical ($^{\bullet}$O$_2$Hb-PFe^{IV} = O) is reduced back to ferri heme, induced by PH. The three investigated

PHs all provoke an identical increase of the rate of decay of the ferryl heme. During this process only a minor amount of PH was converted and therefore the ferryl moiety of Hb, in contrast to compound II of HRP, seems not able to abstract an electron from PH, as we have discussed previously [5]. This might be caused by the inaccessibility of the porphyrin moiety for PH. During decay of ferrylHb oxygen is consumed. The initial increased oxygen consumption in the presence of MaPZ and CPZ (Fig. 5) can be explained by this increasing effect of PHs on ferrylHb decay. The accelerated decomposition of ferrylHb induced by PHs yields a higher turnover of the cycle metHb–ferrylHb (Scheme 1), leading to increased H_2O_2 consumption, independent of which PH is added (Fig. 4). The stimulated metHb–ferrylHb cycle in the presence of PH will also result in increased formation of protein radicals and thereby in a faster consumption of oxygen. Thus, PHs not only reduce the protein radical but in this way also increase the rate of formation of the protein radical. The first process is dependent on the electronic properties of PH, whereas the second is not. The fact that CPZSO does neither increase H_2O_2 consumption nor influence oxygen consumption is in agreement with the inability of CPZSO to induce ferrylHb decay.

To study the behaviour of metHb, modified by the H_2O_2 induced reactions, experiments were repeated in the same incubation mixture. The catalytic conditions to oxidize CPZ are improved as appears from the faster CPZ conversion (Fig. 6). The improved CPZ oxidation is not especially due to modifications provoked by H_2O_2, because pretreatment of metHb with H_2O_2 only results in small increase in rate of CPZ conversion (Fig. 6). Possibly also covalent binding of $CPZ^{+\bullet}$ near the reactive site and/or a conformation change induced by this binding, is involved. In the second reaction a higher CPZSO yield and loss of covalent binding is observed. This might be caused by saturation of available binding sites on the apoprotein. The decreased oxygen consumption during the second reaction (Fig. 7) can also be explained by saturation of available oxidation sites on the apoprotein.

Resuming, the oxidation of PH by metHb in the presence of H_2O_2 is an example of participation of protein radicals in substrate oxidation. Protein radicals have been detected in the reactions of other hemoproteins with peroxides, like cytochrome *c* peroxidase [22] but also cyclooxygenase from ram seminal vesicles [23]. It is possible that protein radicals more generally participate in substrate oxidations.

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