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ACTIVATION OF CHLORPROMAZINE BY THE MYELOPEROXIDASE SYSTEM OF THE HUMAN NEUTROPHIL

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Abstract—The univalent oxidation of chlorpromazine (CPZ) by the myeloperoxidase (MPO–H_2O_2 system led to the formation of a cation free radical ($CPZ^{+\cdot}$) which was observed optically at 527 nm. CPZ protected MPO against loss of catalytic activity when co-oxidized in a MPO–Cl^-–H_2O_2 system. Due to the stability of $CPZ^{+\cdot}$ either further oxidation, or reduction back to the mother compound, become important mechanisms for disappearance of the free radical. Thus, the rate of formation and decay of $CPZ^{+\cdot}$ were higher in the presence of Cl^- than in its absence, since the radical can also be oxidized further by hypochlorous acid (HOCl), which is formed in the MPO–Cl^-–H_2O_2 system. Decay of $CPZ^{+\cdot}$ can also be due to electron acceptance from ascorbic acid or oxygenated haemoglobin (HbO_2), resulting in regeneration of CPZ. When $CPZ^{+\cdot}$ was generated in the MPO–H_2O_2 system, addition of HbO_2 resulted in a sudden decrease in $CPZ^{+\cdot}$ absorbance at 527 nm and a concomitant formation of metHb. When HbO_2 was not added, the decay of $CPZ^{+\cdot}$ was much slower. CPZ (in the absence of the MPO system) also stimulated the oxidation of HbO_2 in the presence of 20 μM H_2O_2, but this reaction was considerably slower than when $CPZ^{+\cdot}$ (generated by the MPO system) was allowed to react directly with HbO_2. These results suggest that HbO_2 was oxidized by $CPZ^{+\cdot}$. To study the effect of CPZ intermediates, thyroglobulin (TG) was used as a model polypeptide. Chlorinated oxidants formed in the MPO system (in the absence of CPZ) induced TG peptide bond splitting. In contrast, CPZ metabolites generated by the MPO system (in the absence of Cl^-) induced polymerization of TG, as revealed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Phenothiazines [of which chlorpromazine (CPZ†) is a prototype] and their metabolites are distributed into most body tissues, with high concentrations being reached in the brain, lungs, liver, kidneys and spleen [1]. Numerous possible metabolites of CPZ have been postulated and many of them have actually been isolated from human urine [2]. Oxidative pathways for CPZ and its metabolites, include N-oxidation of tertiary amines, oxidative deamination, sulphoxidation and aromatic ring hydroxylation [3].

The univalent oxidation of CPZ in acid solution to a free radical intermediate was clearly demonstrated in a variety of EPR studies [4, 5]. The same free radical was also produced during oxidation by horseradish peroxidase and hydrogen peroxide and was found to be identical to the red intermediate observed optically at 530 nm [6].

Although CPZ is employed primarily in the treatment of psychiatric disorders, it has been used occasionally as a free radical scavenger and antioxidant. For example, CPZ can inhibit lipid peroxidation both *in vivo* [7] and *in vitro* [8]. Histochemically, CPZ has been shown to cause diminution of lipofuscin (age pigment) in the nerve cells of the rat brain [9]. Furthermore, the degree of tubular cellular damage decreased in response to mannitol and CPZ during reperfusion of ischaemic rabbit kidneys [10]. A favourable effect has also been achieved by adding antioxidant steroids, CPZ and allopurinol to the solution in which kidneys were stored until transplanted [11].

When stimulated, phagocytes (neutrophils, macrophages) immediately consume large quantities of oxygen, which is transformed almost quantitatively into superoxide anion radicals [12]. Dismutation of superoxide leads to the formation of H_2O_2. The cytotoxicity of H_2O_2 is considerably enhanced in the presence of MPO, which is simultaneously released from the azurophilic granules into the phagocytic vacuoles. In the presence of halides, highly reactive products, such as hypohalous acids or halogens may be formed [13]. MPO has been shown to have both antimicrobial and cytotoxic properties [13]. Cytotoxicity can be induced by catalytically active MPO released into the extracellular environment after neutrophil stimulation [14]. In fact, it has been shown that the MPO system can induce glomerular injury when MPO, followed by H_2O_2 in a chloride-containing solution was infused into the renal artery of rats [15].

Conflicting results regarding the mechanism of CPZ-stimulated metHb formation have been reported. Okano *et al.* [16] postulated that the accelerated oxidation of HbO_2 (in human erythrocyte suspensions and haemolysates) by phenothiazines was caused by radical cations. Kelder *et al.* [17], who have used purified haemoglobin in their studies, also

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† Abbreviations: CPZ, chlorpromazine; $CPZ^{+\cdot}$, chlorpromazine cation free radical; HbO_2, oxygenated haemoglobin; metHb, methaemoglobin; MPO, myeloperoxidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TG, thyroglobulin.

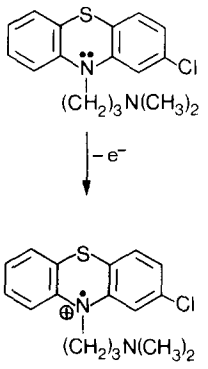

Fig. 1. Structural formulae of chlorpromazine (CPZ) and its 1-electron oxidation product, the cation free radical ($CPZ^{+\cdot}$).

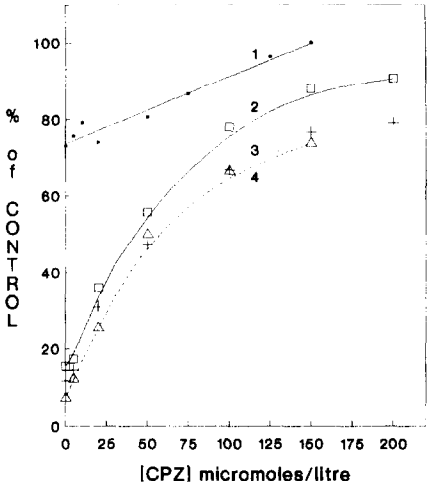

Fig. 2. The effect of Cl^-- and CPZ-derived species on MPO catalytic activity. To each of a series of solutions of 1.3 μM MPO in 50 mM phosphate buffer (pH 6) containing different concentrations of NaCl were added increasing concentrations of CPZ. Reactions were initiated with 500 μM H_2O_2. After 5 min of reaction, 100-μL aliquots were passed through Sephadex G-25 columns. The MPO-containing fractions were analysed for peroxidase activity (guaiacol assay) and expressed as per cent of activity of the same concentration of unreacted MPO. Curves 1, 0 mM NaCl; 2, 10 mM NaCl; 3, 50 mM NaCl; 4, 150 mM NaCl. Each data point represents the average of triplicate analyses.

observed stimulation of HbO_2 auto-oxidation in the presence of CPZ. Since they did not observe transformation of CPZ to the sulphoxide, a mechanism in which CPZ interacts with HbO_2 at a site other than the haem iron was proposed. Such an interaction could distort the iron–oxygen bond sufficiently to facilitate electron transport from iron (II) to oxygen. In the present study, the MPO–H_2O_2 system was used to generate $CPZ^{+\cdot}$ (Fig. 1) and the interaction of HbO_2 with the radical was monitored spectrophotometrically. Since H_2O_2 also interacts with HbO_2 and in the presence of CPZ will stimulate metHb formation, experimental conditions were chosen to minimize this effect to the advantage of the $CPZ^{+\cdot}$–HbO_2 interaction. To study the effects of reactive intermediates on protein structure, TG was used as a model polypeptide. For this purpose, TG seems to be ideally suited, since it is also cleaved and polymerized by the thyroid peroxidase–H_2O_2–I^- system during thyroid hormone synthesis (e.g. Refs 18 and 19).

MATERIALS AND METHODS

Chlorpromazine hydrochloride was obtained from Maybaker (SA) (Pty) Ltd. Sodium hypochlorite (approx. 1 N in 0.1 N NaOH) was from BDH. HOCl was obtained by adjusting NaOCl to pH 6.2 with dilute H_2SO_4.

Preparation of proteins. MPO was isolated from human neutrophils as described previously [20] and had purity indexes (A_{428nm}/A_{280nm}) ranging from 0.75 to 0.8. Its concentration was calculated using an absorption coefficient of $89 \times 10^3\,M^{-1}\,cm^{-1}$ per haem at 428 nm [21]. Haemoglobin was isolated from outdated red cells and its concentration determined using an absorption coefficient of $3.0 \times 10^4\,M^{-1}\,cm^{-1}$ at 522 nm for the tetrameric molecule [17]. Bovine TG was prepared by linear sucrose gradient density ultracentrifugation [22].

Generation of $CPZ^{+\cdot}$ and spectrophotometric analyses. $CPZ^{+\cdot}$ was generated by adding H_2O_2 to solutions of MPO in either 50 mM phosphate buffer (pH 6) or 50 mM acetate buffer (pH 4). Formation and decay of $CPZ^{+\cdot}$ and modifications of haem spectra during CPZ oxidation were monitored on a Cary 219 recording spectrophotometer.

Preparation of samples for peroxidase activity. To remove reactants and products from MPO, aliquots (100 μL) of reaction mixtures were passed through Sephadex G-25 columns (2 × 1.3 cm) and peroxidase activity was measured by the guaiacol assay.

SDS–PAGE of TG polypeptides. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of TG polypeptides was performed on 4% slab gels (3 mm thick) according to Laemmli [23], but without a stacking gel. A constant current of 50 mA was applied and 25 mg Coomassie Brilliant Blue R-250/L was added to the cathode buffer for direct visualization of polypeptides during electrophoresis [24].

Further experimental details are described in the Results section.

RESULTS

Effect of CPZ on MPO catalytic activity during turnover

Incubation of 1.3 μM MPO and 500 μM H_2O_2 for 5 min at 20°, resulted in a loss of more than 25% of the MPO activity as determined by the guaiacol assay after MPO had been separated from excess products and reagents by Sephadex G-25 (Fig. 2; curve 1). Incubation in the presence of CPZ show a positive correlation between CPZ concentration and protection of peroxidase activity. At a CPZ concentration of 150 μM, no loss of activity was encountered during the incubation (curve 1). Incubations of MPO and H_2O_2 in the presence of chloride, resulted in considerable loss of peroxidase

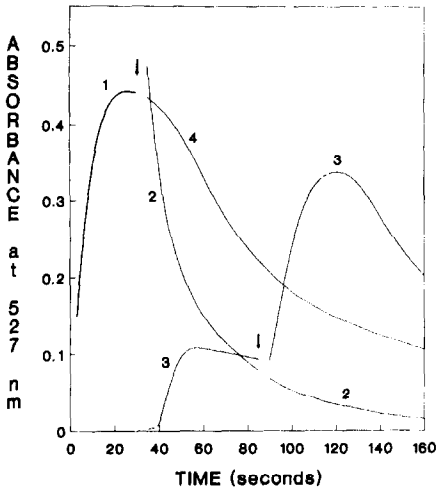

Fig. 3. The effect of reagent HOCl and ascorbic acid on the decay of $CPZ^{+\cdot}$. $CPZ^{+\cdot}$ was generated in a 50 mM acetate buffer (pH 4) containing 1.3 μM MPO and 200 μM CPZ. Reactions were initiated with 200 μM H_2O_2. Curve 1: Formation of $CPZ^{+\cdot}$. Curve 2: HOCl (50 μM in mixture) was added 30 sec after initiation of the reaction (indicated by the arrow) and recording continued 5 sec later; at 85 sec, fresh MPO and H_2O_2 were added (indicated by the second arrow), with no response. Curve 3: Ascorbic acid (50 μM in mixture) was added 30 sec after initiation of the reaction; at 85 sec, H_2O_2 was added. Curve 4: Spontaneous decay of CPZ^+.

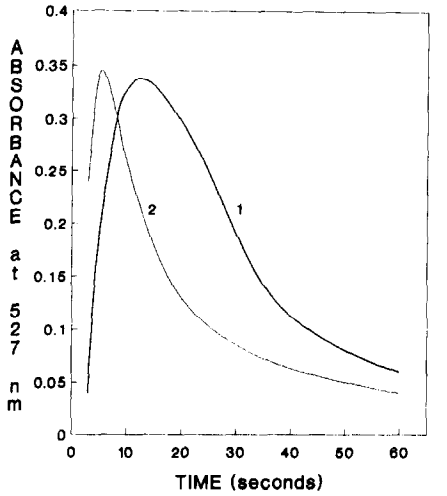

Fig. 4. The effect of Cl^-, co-oxidized with CPZ, on the rate of formation and decay of $CPZ^{+\cdot}$. The reaction mixtures contained 1.3 μM MPO and 200 μM CPZ in 50 mM phosphate buffer (pH 6) in the absence (curve 1) and in the presence of 150 mM NaCl (curve 2). Recordings of the formation and decay of $CPZ^{+\cdot}$ were started 3 sec after adding H_2O_2 (200 μM in mixture).

activity (curves 2, 3 and 4). After incubation in the presence of 150 mM NaCl (without CPZ), only 8% of the original activity could be recovered (curve 4). Protection of MPO in the chloride-containing incubation mixtures by increasing concentrations of CPZ is indicated by curves 2, 3 and 4. Only 10% of the MPO activity was lost when 200 μM CPZ was co-oxidized with 10 mM NaCl in our incubation system (curve 2). The curves for 50 and 150 mM NaCl were very similar (curves 3 and 4). About 80% of the peroxidase activity could be recovered in the 50 mM NaCl/200 μM CPZ reaction mixture (curve 3).

Effect of electron donors and electron acceptors on $CPZ^{+\cdot}$ decay

The decay of $CPZ^{+\cdot}$, monitored at 527 nm, is demonstrated in Fig. 3. This experiment was performed at pH 4, where $CPZ^{+\cdot}$ had a considerably longer lifetime than at pH 6. Thirty sec after adding 200 μM H_2O_2 to a solution containing MPO and 200 μM CPZ (curve 1), reagent HOCl to a concentration of 50 μM was added and thoroughly mixed. Recording of A_{527} was continued 5 sec later and showed initially an increase in absorbance (curve 2), which indicated additional formation of $CPZ^{+\cdot}$. Presumably HOCl was added at a stage where the rate of disappearance of $CPZ^{+\cdot}$, due to its further oxidation, was just higher than its formation from the parent compound. The red colour, however, immediately faded away at a higher rate (curve 2) than the control (curve 4) to which no HOCl had been added. It is known that HOCl does not accumulate in biological systems, but instead almost instantaneously disappears in multiple reactions with available substrates [25], e.g. by degrading MPO haem and consequently inactivating the enzyme. However, adding fresh MPO, followed by H_2O_2 at 85 sec, could not regenerate the red colour of $CPZ^{+\cdot}$, which implies that the disappearance of $CPZ^{+\cdot}$ was due to its further oxidation by HOCl. Alternatively, when 50 μM ascorbic acid was added, 30 sec after initiating generation of $CPZ^{+\cdot}$, the red colour vanished almost instantaneously (curve 3). After a lag period of a few seconds, the reducing potential of the ascorbic acid was overcome and the absorbance at 527 nm increased again. Since H_2O_2 was now the limiting factor, 200 μM was added, 85 sec after initiating the reaction. This led to the formation of additional $CPZ^{+\cdot}$ (curve 3) with maximum A_{527} of about 80% of that of the first maximum (curve 1). This suggests that $CPZ^{+\cdot}$ was reduced back to the mother substance by accepting electrons from ascorbic acid.

The profiles depicted by Fig. 4 show that co-oxidation of physiological concentrations of chloride in the MPO–CPZ–H_2O_2 system had a profound effect on both the rate of generation of $CPZ^{+\cdot}$ and also its decay (curve 2). The effect of 150 mM NaCl is shown here, but the same trend can also be demonstrated for lower NaCl concentrations, albeit to a lesser extent.

Formation of metHb

Scan 1 of Fig. 5B shows a UV scan of 2 μM HbO_2 between 450 and 700 nm at pH 4. On addition of 20 μM H_2O_2, oxidation of HbO_2 can be noted as a progressive decrease in absorbance at 540 and 576 nm in curves 2, 3 and following which were recorded at 1 min intervals. In the presence of 100 μM CPZ, formation of metHb was much faster (Fig. 5A). Scan 2 was recorded 15 sec after adding 20 μM H_2O_2 to the HbO_2/CPZ mixture and scans

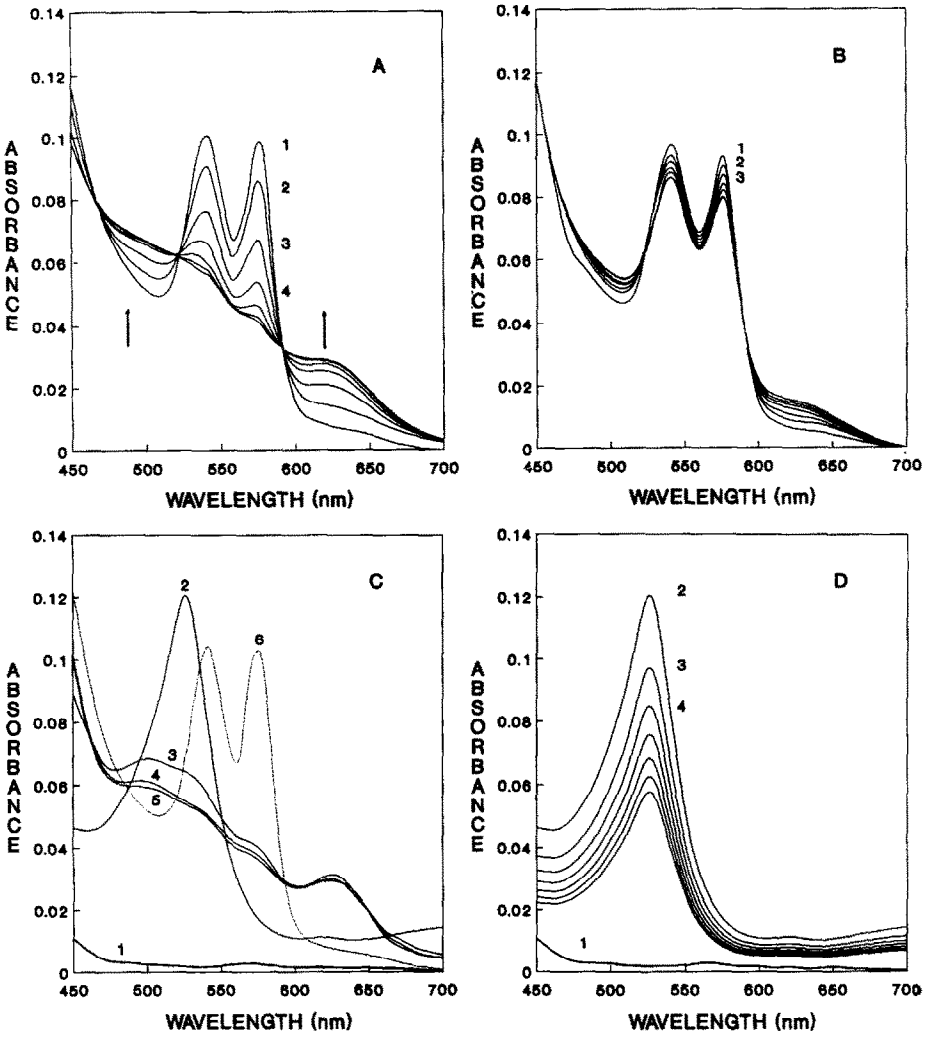

Fig. 5. Oxidation of HbO_2 in 50 mM acetate buffer (pH 4). (A) Absorbance spectra of HbO_2 in the presence of 100 μM CPZ: scan 1, before H_2O_2 addition; scan 2, 15 sec after adding H_2O_2 (20 μM in mixture); scans 3 and following recorded at 1 min intervals. (B) Absorbance spectra of HbO_2 in the absence of CPZ: scan 1, before H_2O_2 addition; scan 2, 1 min after adding H_2O_2 (20 μM in mixture); scans 3 and following recorded at 1 min intervals. (C) Scan 1, spectrum of 0.4 μM MPO + 100 μM CPZ before H_2O_2 addition; scan 2, 1 min after adding H_2O_2 (20 μM in mixture); scan 3, 15 sec after adding HbO_2 (2 μM in mixture); scans 4 and 5 recorded at 1 min intervals; scan 6, reference spectrum of 2 μM HbO_2. (D) Scans 1 and 2 same as in (C) and then at 1 min intervals for scans 3 and following.

3, 4 and following were recorded at 1 min intervals. After about 5 min, virtually all HbO_2 had been converted to metHb. Scan 1 (Fig. 5A) and scan 1 (Fig. 5B) appear to be similar indicating that CPZ did not have a direct effect on the HbO_2 spectrum. In Fig. 5C, $CPZ^{+\cdot}$ was generated by adding 20 μM H_2O_2 to a mixture of 0.4 μM MPO and 100 μM CPZ (scan 2). Scan 3 was recorded 15 sec after adding 2 μM HbO_2 to the solution and shows a sudden drop in absorbance at 527 nm which was coupled to a decrease in HbO_2 absorbance at 540 and 576 nm. For reference purposes, the UV scan of 2 μM HbO_2 was superimposed on the other spectra (scan 6). Scans 4 and 5 were recorded at 1 min intervals. Scan 1 shows the negligible absorbance of 0.4 μM MPO in the operational wavelength. Figure 5D depicts the decay of $CPZ^{+\cdot}$ without addition of HbO_2. The recordings, which were made at 1 min intervals, shows a much slower decrease in 527 nm absorbance with time than in Fig. 5C.

Effect of CPZ oxidation products on a model polypeptide

Figure 6 shows SDS–PAGE patterns of TG under non-reducing conditions. TG was incubated in different reaction systems for 10 min at 37° before electrophoresis. Lane 1 represents the electrophoresis pattern of a control TG sample incubated in the presence of 133 nM MPO, 150 mM NaCl and 200 μM CPZ, i.e. the full system with the exception of H_2O_2. In lane 2, TG was incubated with 133 nM MPO and 500 μM H_2O_2. Omission of MPO or H_2O_2, or both,

did not change the polypeptide pattern of lane 2 which appears to be identical to lane 1. The TG half-molecule (M_r = 330 kDa) as well as higher polymers near the top of lanes 1 and 2 are clearly visible. In lanes 3 and 4, the oxidizing mixture contained 10 and 50 mM NaCl, respectively. The Coomassie Blue stains have a more diffuse character and additional smaller bands are also visible. In addition, the stains at the ion front are somewhat more intense in lanes 3 and 4 than in the other lanes. For lanes 5 and 6, the oxidizing mixtures contained 50 and 200 μM CPZ, respectively. The stains at the half-molecule position are clearly of a lower intensity than those in the controls (lanes 1 and 2). In addition, less of the half-molecule was present in lane 6 (200 μM CPZ) than in lane 5 (50 μM CPZ). This was coupled to a higher intensity of the stains near the top of the gel (lanes 5 and 6). In the reaction mixture of lane 7, 10 mM NaCl and 50 μM CPZ were co-oxidized, while for lane 8, the reaction mixture contained 10 mM NaCl and 200 μM CPZ. The polypeptide patterns in lanes 7 and 8 seem to be comparable to those of lanes 5 and 6 which did not contain chloride in the reaction mixtures.

DISCUSSION

Modulation of peroxidase activity by CPZ and metabolites

It is well known that chlorinated oxidants (e.g. HOCl) generated in the MPO–H_2O_2–Cl^- system can destroy MPO catalytic activity. In Fig. 2 it is shown that more than 80% of the peroxidase activity was lost after the MPO–Cl^- reaction had been initiated with H_2O_2 in the absence of CPZ. When, however, CPZ was co-oxidized in the MPO–Cl^-–H_2O_2 system, peroxidase function was protected and the extent of protection was dependent upon the concentration of CPZ used. The mechanism for the protective action of CPZ towards MPO in the MPO–Cl^-–H_2O_2 system could be two-fold:

(i) Since CPZ is an excellent electron donor, it will favourably compete with Cl^- as MPO substrate and thus limit production of the damaging HOCl.
(ii) At least some of the HOCl which may still be formed will be scavenged by CPZ and/or its intermediate oxidation products, since reagent HOCl interacted with $CPZ^{+\cdot}$ (Fig. 3; curve 2).

CPZ itself or its oxidation products, at concentrations which we have used, does not appear to be detrimental to MPO, since an adequate concentration of CPZ could prevent loss of catalytic activity fully (Fig. 2; curve 1). Some loss of peroxidase activity was, however, always encountered in the Cl^-/CPZ mixed system. This suggests that some chlorinating oxidants were formed, irrespective of the CPZ concentration. It is evident from Fig. 2 that the maximum amount of protection afforded by CPZ was lower at higher Cl^- concentrations. It is also possible that the scavenging reaction of CPZ and/or its metabolites with HOCl could produce secondary chlorinating oxidants (e.g. chloramines) which could have been responsible for some impairment of MPO catalytic activity.

Formation and decay of $CPZ^{+\cdot}$

The first one-electron transfer product of CPZ ($CPZ^{+\cdot}$) was formed during oxidation by MPO and H_2O_2 and could be monitored spectrophotometrically at 527 nm. Since CPZ is poorly soluble at neutral or alkaline pH, experiments were performed at pH 6, or at pH 4 when greater stability of the cation radical was required. It was reported that $CPZ^{+\cdot}$ is stabilized to the extent of protonation of its alkyl nitrogen side chain [26]. Our experimental conditions should, however, not be considered as entirely biologically irrelevant, since the intravacuolar pH of neutrophils has been reported to be less than 6 within 1 hr after initiation of phagocytosis [27]. Also, the pH beneath adherent activated macrophages has been reported to be 5 or less [28].

Oxidation of CPZ can be effected by HOCl, which is formed in the MPO–Cl^-–H_2O_2 system. It was found that reagent HOCl accelerated $CPZ^{+\cdot}$ decay (Fig. 3; curve 2). When Cl^- was included in the MPO–CPZ–H_2O_2 system, both the rate of formation of $CPZ^{+\cdot}$ and also its decay was increased (Fig. 3). Accelerated decay of $CPZ^{+\cdot}$ will thus be due to its additional oxidation by HOCl formed by the MPO–Cl^-–H_2O_2 component of the mixed system.

In the presence of ascorbic acid, the $CPZ^{+\cdot}$ yield was very low, which could have been due to substrate inhibition. The results of Fig. 3, however, indicated that ascorbic acid accelerated $CPZ^{+\cdot}$ decay. This suggests that $CPZ^{+\cdot}$ was reduced back to CPZ by accepting electrons from ascorbic acid. Indeed, it could be demonstrated that re-addition of H_2O_2, after decay of $CPZ^{+\cdot}$ had been induced by ascorbic acid, regenerated $CPZ^{+\cdot}$ (Fig. 3; curve 3) to about 80% of that of the initial formation (curve 1). This is in accordance with the suggestion made by Piette *et al.* [6] that ascorbic acid does not inhibit formation of $CPZ^{+\cdot}$, but instead acts as a radical trap once the free radical was formed. They observed that the reaction of $CPZ^{+\cdot}$ with ascorbic acid was faster than the further enzymatic oxidation of the free radical.

Interaction of $CPZ^{+\cdot}$ with HbO_2

Figure 5C clearly demonstrates that $CPZ^{+\cdot}$ interacts with HbO_2. Within 15 sec after adding HbO_2 to the free radical-generating system, virtually all absorbance due to $CPZ^{+\cdot}$ vanished. This was coupled to an almost complete conversion of HbO_2 to metHb. These observations suggest that $CPZ^{+\cdot}$ was reduced back to the mother substance by accepting electrons from HbO_2 which was consequently oxidized to metHb. The contribution of the absorbance of MPO (in the $CPZ^{+\cdot}$-generating system) towards the total absorbance of the system was negligible in the operational wavelength range. In the classical pathway for peroxidase action, higher oxidation states of the enzyme are formed in the presence of H_2O_2 (compounds I and II) which are the actual oxidants. To eliminate the possibility that HbO_2 could have been oxidized by these higher oxidation states of MPO, a control experiment was performed in which CPZ was omitted from the reaction mixture which contained only MPO, HbO_2 and H_2O_2 at relevant concentrations. It was found that the rate of conversion of HbO_2 to metHb in such a system was comparable to the rate of conversion in a system where H_2O_2 was added to a mixture containing only HbO_2 (Fig. 5B). Another possibility exists; it is

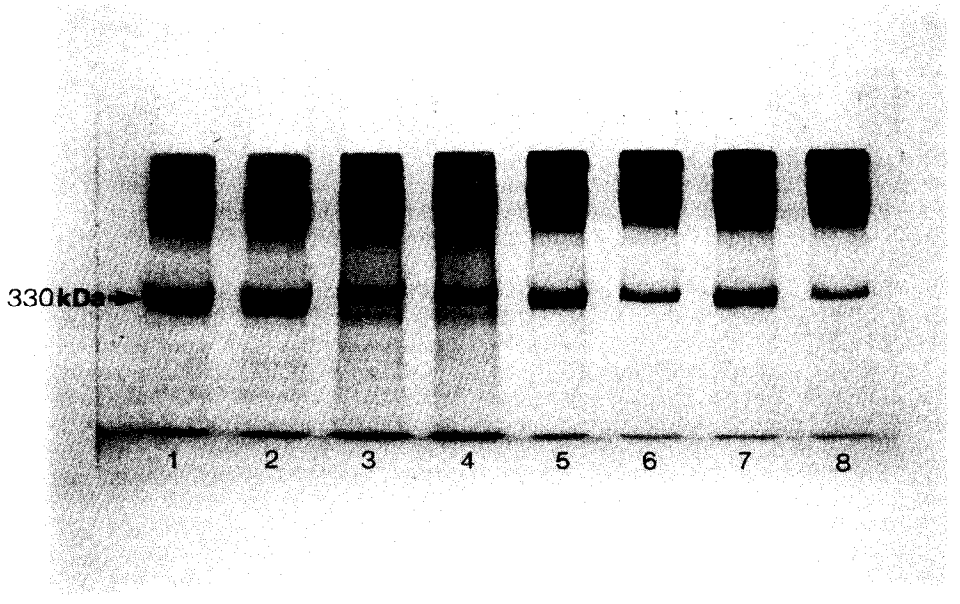

Fig. 6. SDS–PAGE (4% gel) demonstrating the effect of CPZ and Cl^- on TG polypeptide composition in the MPO–H_2O_2 system. TG (2 mg/mL) was incubated with 133 nM MPO in 50 mM phosphate buffer (pH 6). The reaction was started by the addition of H_2O_2 where applicable (final concentration 500 μM) and proceeded for 10 min at 37°. Before electrophoresis, the samples were treated with SDS (final concentration 1%) and 50-μL aliquots applied to the gel. A constant current of 50 mA was applied until satisfactory separation of polypeptides was achieved. The gel was stained with 0.02% Coomassie Brilliant Blue R-250 and destained with acetic acid/methanol/water (1:2:7). The following mixtures were analysed: Lanes 1, TG + MPO + 150 NaCl + 200 μM CPZ; 2, TG + MPO; 3, TG + MPO + 10 mM NaCl; 4, TG + MPO + 50 mM NaCl; 5, TG + MPO + 50 μM CPZ; 6, TG + MPO + 200 μM CPZ; 7, TG + MPO + 50 μM CPZ + 10 mM NaCl; 8, TG + MPO + 200 μM CPZ + 10 mM NaCl. Reaction mixture of lane 1 was incubated without H_2O_2 and of lanes 2–8 incubated with 500 μM H_2O_2.

known that H_2O_2 can react with HbO_2 and metHb to form ferrylHb [29] (see also Fig. 5B) which is formally equivalent to compound II of peroxidases. FerrylHb (oxidation state IV) can oxidize CPZ to the sulphoxide [17] and is itself reduced to metHb. Stimulation of metHb formation in the presence of CPZ could be demonstrated in Fig. 5A, but it is also obvious that this rate of metHb formation was much slower than that shown in Fig. 5C where virtually all HbO_2 was converted within 15 sec. Since $CPZ^{+\cdot}$ is a precursor of the sulphoxide, the question arises why the free radical did not accumulate in our HbO_2–CPZ–H_2O_2 system. Kelder *et al.* [17] reported that CPZ enhanced the auto-oxidation of HbO_2 without being transformed itself. During their incubation, spectral changes suggestive of transformation of HbO_2 into metHb were observed even in the absence of CPZ. Two auto-oxidative mechanisms are proposed for HbO_2; one involving loss of superoxide and the other acquisition of an electron and loss of H_2O_2 [30]. Thus, it is likely that small amounts of ferrylHb could have been formed during the auto-oxidative process. FerrylHb in turn can oxidize CPZ to the cation radical ($CPZ^{+\cdot}$), which could accept an electron from HbO_2 to be reduced back to CPZ again. In this way, CPZ could stimulate the auto-oxidation of HbO_2 apparently without being transformed itself. Under conditions of high H_2O_2 excess (200 μM or more), some accumulation of $CPZ^{+\cdot}$ did occur in our HbO_2 reaction mixture. It was further noted that the typical UV spectrum of $CPZ^{+\cdot}$ could only be observed after most HbO_2 had been converted to metHb.

The inhibition of lipid peroxidation by phenothiazines has been related to both their antioxidating and iron chelating properties [31]. It is known that iron and iron compounds catalyse ·OH production and lipid peroxidation. Haemoglobin itself has even been described as a Fenton reagent [32]. However, it has since been reported that free iron, derived from haemoglobin, may be the proximate toxic species [33]. If $CPZ^{+\cdot}$ can oxidize Fe^{2+} in HbO_2, it is likely that it will also oxidize free iron ions (which it can chelate in any case). It is known that $CPZ^{+\cdot}$ is produced in the reverse reaction, i.e. Fe^{3+} can oxidize CPZ in acid medium to the free radical [4, 5]. The following equilibrium reaction may be written:

$$CPZ + Fe^{3+} \rightleftharpoons CPZ^{+\cdot} + Fe^{2+}.$$

If $CPZ^{+\cdot}$ is produced by other means (enzymatic or during HbO_2 oxidation and subsequent ferrylHb formation), the equilibrium will be driven to the left and thus Fe^{2+} which is necessary for ·OH production in the Fenton reaction, will be a limiting factor. Such an inhibition of ·OH production may be relevant, since phenothiazines are transported to and released from the brain by means of erythrocytes [34]. It is interesting to note that CPZ has been used to

decrease reperfusion injury of ischaemic rabbit kidneys [10]. In this case CPZ has, however, been proposed to act as an ·OH scavenger.

Thyroglobulin as a model polypeptide to evaluate the effect of destructive CPZ metabolites

The opposite effects which HOCl, and CPZ metabolites (both generated in the MPO–H_2O_2 system) have on TG polypeptide structure, is clearly demonstrated in Fig. 6. HOCl caused peptide bond splitting (fragmentation) of TG (lanes 3 and 4), while CPZ metabolites induced polypeptide polymerization (lanes 5 and 6). These transformations must be due to Cl^-- or CPZ-derived species, respectively, since the MPO–H_2O_2 system had no obvious direct effect on TG polypeptide structure (lane 2). In mixed Cl^-/CPZ systems, the SDS–PAGE patterns were more comparable to those containing CPZ without Cl^-. This can be explained by the fact that CPZ is a superior electron donor compared to Cl^- and thus the production of HOCl, as the potential peptide bond splitter, will be limited. Also HOCl, nevertheless formed, can be scavenged by CPZ and/or its metabolites. When CPZ (or other promazines) are exposed to near-UV light they undergo photoionization giving rise to $CPZ^{+\cdot}$ and the hydrated electron [35]. Ocular opacities described in patients under high doses of these drugs and exposed to sunlight, could be due to cross-linking of lens proteins [36]. It has also been observed that *in vitro*, CPZ sensitizes the photodynamic oxidation of sulphydryl groups of some biological molecules [37]. Thus, reactive CPZ intermediates formed in the MPO system may have similar effects in cross-linking TG polypeptide chains. Electrophoresis was performed under non-reducing conditions, since we did not want to reduce disulphides again which could form during interaction of CPZ metabolites with TG sulphydryl groups. Although reduction of TG with 2-mercaptoethanol significantly decreased the concentration of polymerization products in all lanes (results not shown), it was also evident that not all polymerization induced by CPZ metabolites was due to disulphide formation. This suggests that other "non-reducible" bonds were also induced by CPZ metabolites.

Various drugs can undergo co-oxidation in the MPO–Cl^-–H_2O_2 system and have been shown to serve as electron donors to peroxidase. The latter reaction may lead to the formation of electron-deficient drug metabolites, such as free radicals. Depending on the nature of the free radicals or the conditions under which they were generated, reaction with the MPO molecule itself or reaction with any neighbouring molecule is possible. For example, the one-electron oxidation of paracetamol leads to the formation of phenoxy free radicals which can polymerize to form 2,2′-biphenols and higher polymers [38]. The reactive intermediates derived from paracetamol oxidation, however, did not impair the catalytic function of MPO, nor did it induce polypeptide modification of TG present in the reaction medium [39]. Reactive intermediates generated from the antituberculous drug, isoniazid, caused extensive damage to MPO haem [40], but it could not induce polypeptide modification of TG. CPZ reactive intermediates, as shown in the present study, had a different effect; the catalytic function of MPO was not impaired, while the polypeptide composition of TG was significantly modified during the MPO-catalysed reaction. It is proposed that this feature of CPZ may play a role in the toxicological manifestations of the drug.

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