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IS HEMOGLOBIN A CATALYST FOR SULFOXIDATION OF CHLORPROMAZINE?

AN INVESTIGATION WITH ISOLATED PURIFIED HEMOGLOBIN AND HEMOGLOBIN IN MONOOXYGENASE AND PEROXIDASE MIMICKING SYSTEMS

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Abstract—The possible role of hemoglobin in the sulfoxidation of chlorpromazine is still a controversial subject. Therefore this sulfoxidation was investigated with purified oxyhemoglobin and methemoglobin under various conditions: (i) in phosphate buffer pH 6.5; (ii) in monooxygenase mimicking systems with electron donors like ascorbic acid and NADPH, the last, with and without an electron carrier like methylene blue and cytochrome *c* reductase; (iii) in the presence of H_2O_2. Only in the presence of H_2O_2 chlorpromazine was converted into chlorpromazine sulfoxide in a considerable amount. This so-called peroxidase activity of hemoglobin appeared not to be based on a Fenton-type reaction. An oxidized reactive form of hemoglobin (i.e. ferrylhemoglobin) is responsible for the sulfoxidation. In the other systems only with ascorbic acid some chlorpromazine sulfoxide was produced. This is probably due to the production of H_2O_2 and the subsequent peroxidase activity of hemoglobin. Chlorpromazine enhanced the autoxidation of oxyhemoglobin, without being transformed itself.

Chlorpromazine (CPZ)† is an important representative of the widely used phenothiazine neuroleptics. This class of anti-psychotics consists of an extended range of structurally related drugs which vary in substitution at the 2 and 10 positions. The metabolism of phenothiazines is complex and species dependent. Several major biotransformation routes are observed: (i) sulfoxidation, (ii) aromatic ring hydroxylation, (iii) N-mono- and N-didemethylation, (iv) N-oxidation. These processes may occur in combination and may be followed by glucuronidation or sulfatation [1]. Different enzymes, located in different tissues (liver, gutwall, lung) are responsible for these transformations [2–4]. In humans a significant portion of CPZ is metabolized to chlorpromazine sulfoxide (CPZSO) [5], which is pharmacologically inactive [6].

Minder *et al.* [7] and Traficante *et al.* [8] reported considerable sulfoxidation of CPZ in whole blood *in vitro*. They attributed this sulfoxidizing activity to hemoglobin (Hb). Kaul *et al.* [9] also found sulfoxidation induced by whole blood, erythrocytes and erythrocyte lysate but not with solutions of commercial Hb. Traficante *et al.* [8] and Kaul *et al.* [9] assumed a protein-like factor located in plasma that actively inhibits blood catalysed CPZ sulfoxidation. The instantaneous conversion of CPZ into CPZSO in whole blood observed by the above mentioned investigators caused Hawes *et al.* [10] to raise the question whether this is a true physiological event or an artefact introduced during analysis. These investigators showed that the conversion of CPZ into CPZSO in whole blood is largely due to artefacts resulting from the use of alkali in the analytical procedure. They found that CPZ and CPZSO are remarkably stable when added separately to whole blood and incubated at 37° *in vitro*.

The conflicting results on the possible role of Hb in the metabolism of CPZ prompted us to study the reaction of CPZ with purified Hb under various conditions.

Apart from its oxygen transport function, Hb has hydroxylating [11–13], dealkylating [14] and in the presence of H_2O_2 peroxidating properties [15, 16]. These activities are explained by assuming that Hb can be activated to a form which is similar to the active form of cytochrome P-450 [12–14, 17]. In this respect Hb is frequently used as a model for cytochrome P-450. These model systems often contain electron donors like ascorbic acid [13] and NADPH with or without electron carriers like cytochrome *c* reductase [11–14] or methylene blue [12, 13] to complete the monooxygenase system. We investigated the conversion of CPZ into CPZSO in some of these Hb-containing monooxygenase model systems. We also investigated this sulfoxidation of CPZ by Hb in the presence of H_2O_2. Considering the results of Hawes *et al.* [10] the use of alkali in our analytical procedures was strictly excluded.

MATERIALS AND METHODS

CPZSO was a gift from Rhône-Poulenc (Paris, France). CPZ, human Hb, β-NADPH, cytochrome

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† Abbreviations used: CPZ, chlorpromazine; CPZSO, chlorpromazine sulfoxide; Hb, hemoglobin in general; oxyHb, deoxyHb, metHb, and ferrylHb, oxy-, deoxy-, met- and ferrylhemoglobin, respectively.

c reductase (EC 1.6.99.3; type 1; activity as defined by Sigma) were obtained from Sigma Chemical Co. (St Louis, MO). $Na_2HPO_4.2H_2O$ and KH_2PO_4 were obtained from Fluka AG (Buchs, Switzerland). All other chemicals were obtained from E. Merck (Darmstadt, F.R.G.) and were of analytical quality. These compounds were used as purchased. Whole blood was obtained from the Blood Bank (Utrecht, The Netherlands).

Phosphate buffers had a constant ionic strength of 0.1 M and always contained 0.2 mM EDTA. All solutions were prepared with demineralized water purified through a Millipore purification system (15 MΩ water).

Spectrophotometric analyses. Spectrophotometric analyses were performed on a Perkin Elmer Lambda 5 UV/VIS Spectrophotometer.

HPLC analyses. HPLC analyses were performed on a liquid chromatography system built from the following components (Waters Associates): a Model 6000 A solvent delivery system combined with a WISP® Model 710 B autosampler and a Model 440 double channel absorbance detector, set at 254 and 340 nm. Samples of 20 μl were chromatographed on a 5 μm Spherisorb Cyano-column (15 cm × 4.6 mm i.d.; Phase Separation Ltd). 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. Retention times were: CPZ 4.7 min, CPZSO 6.9 min.

Treatment of commercial hemoglobin. The commercial human Hb contained approximately 80% methemoglobin (metHb). A solution of this product in phosphate buffer pH 6.5 was converted to oxyhemoglobin (oxyHb) by adding a slight excess of solid $Na_2S_2O_4$ and subsequent elution with the same buffer over a Sephadex G-25 M column, bedvolume 9.1 ml, Pharmacia (Uppsala, Sweden). Conversion to metHb was performed by adding a two-fold molar excess of $K_3Fe(CN)_6$ and elution over Sephadex G-25 M, as described above. These treatments were performed directly before use.

This commercial Hb was controlled for the following enzymatic activities by the methods indicated: superoxide dismutase (EC 1.15.1.1.) [18], catalase (EC 1.11.1.6) [19] and gluthathion peroxidase (EC 1.11.1.9) [20]. None of these enzymatic activities was observed.

Preparation of purified hemoglobin. OxyHb was isolated from human whole blood according to the method of Eyer *et al.* [21]. The isolated oxyHb gave one band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. It contained no superoxide dismutase, no catalase, and no glutathion peroxidase activity as determined by the above methods. The purified oxyHb was stored at −80°. Directly before use this oxyHb solution was eluted on a Sephadex G-25 M column with phosphate buffer pH 6.5. MetHb was prepared as described for the commercial Hb.

Determination of concentration and percentage oxyhemoglobin. OxyHb and metHb have an isosbestic point at 522 nm. Mixtures of oxyHb and metHb were quantitated at this wavelength using a molar absorptivity of 3.0×10^4/M/cm for the tetrameric molecule [22].

The percentage oxyHb of the total Hb content is calculated from the absorbances (A) at 540 nm and 576 nm:

$$X = \frac{70\,A_{540} - 117\,A_{576}}{169\,A_{576} - 236\,A_{540}} \times 100\%.$$

This equation is based on reported molar absorptivities of oxyHb and metHb at pH 6.5 [23]. It is assumed that oxyHb is converted to metHb only and that the concentration of deoxyhemoglobin (deoxyHb) is negligible under our conditions. When the spectrum had deviated slightly due to turbidity, proper corrections for the absorbances were made.

Incubations. Typical reaction mixtures contained 0.1 mM oxyHb or metHb, 2 mM CPZ in phosphate buffer pH 6.5 in a volume of 1.25 ml. The experiments were performed at pH 6.5, because at pH 7.4 CPZ is poorly soluble. Further details of the composition of incubation mixtures are given in Results. Reactions were started by the addition of oxyHb or metHb and, when applicable, by the addition of H_2O_2. Several control experiments have been performed: (i) omission of oxyHb or metHb; (ii) omission of CPZ, (iii) substitution of oxyHb and metHb by $FeSO_4$ and $FeCl_3$, respectively, to test the involvement of reactions of the Fenton-type in the H_2O_2-containing experiments, and (iv) like (iii) but without H_2O_2.

The reactions were performed in air saturated solutions, in the dark in glass vials of 10 ml placed in an oscillating waterbath thermostated at 37.0 ± 0.05. At certain time intervals samples of 50 μl and 20 μl were taken. The 50 μl samples were brought in 0.45 ml acetonitrile and mixed on a Vortex mixer to stop the reaction by precipitation of Hb. After centrifugation at 15,000 rpm for 5 min, the supernatant was analysed by HPLC. Recovery experiments showed that CPZ as well as CPZSO were extracted over 99% from Hb-containing solutions as compared to control solutions in phosphate buffer without metHb or oxyHb. The 20 μl samples were diluted with buffer to 0.60 ml and absorption spectra from 450–700 nm were recorded directly.

RESULTS

Incubations with oxyhemoglobin and methemoglobin

HPLC analysis of the incubations with oxyHb as well as metHb (both purified and commercial) showed that CPZ metabolites, including CPZSO, were not produced up to 5 hr. However, the CPZ concentration diminished during the first 2 hr of incubation and a final decrease of 5% in the oxyHb and 20% in the metHb containing mixtures was observed. This loss of CPZ was not the result of CPZ transformation but was due to precipitation of CPZ with oxyHb and metHb. Analysis after centrifugation demonstrated that the precipitate contained Hb and CPZ but no CPZ metabolites.

During the incubations with oxyHb spectral changes characteristic for transformation of oxyHb into metHb were observed. In the presence of CPZ this autoxidation is faster than without CPZ (Figs 1 and 2). Commercial oxyHb also showed a faster autoxidation in the presence of CPZ. During the

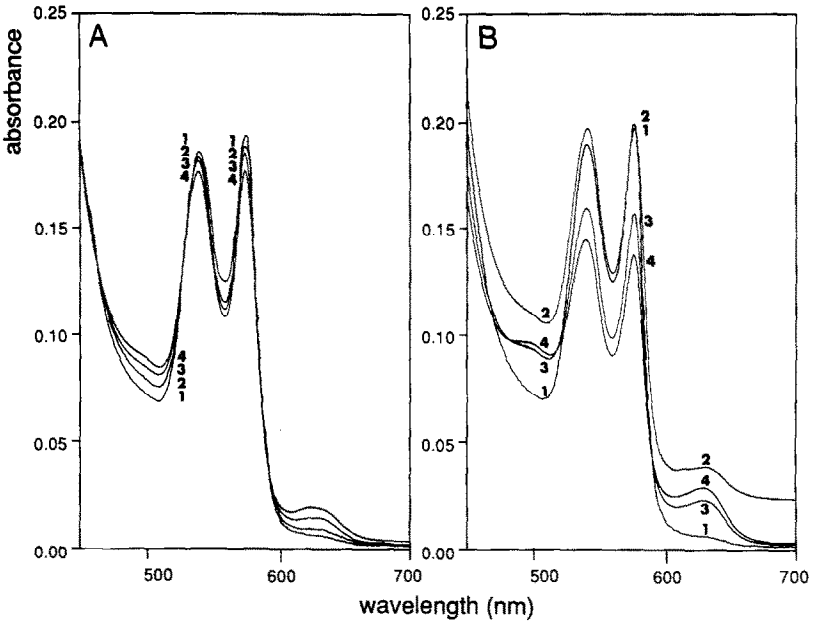

Fig. 1. Visible absorption spectra showing autoxidation of oxyHb in the absence (A) and in the presence (B) of CPZ. OxyHb (0.1 mM) was incubated in phosphate buffer pH 6.5 (ionic strength 0.1 M) in the dark at 37°; 2.0 mM CPZ (B). Spectra were recorded after 30-fold dilution at time intervals: 2 min (1): 1 hr (2); 3 hr (3); 5 hr (4). Spectrum 2B deviates due to turbidity which disappeared after longer incubation time. At the start of the experiment, oxyHb contained 8% metHb.

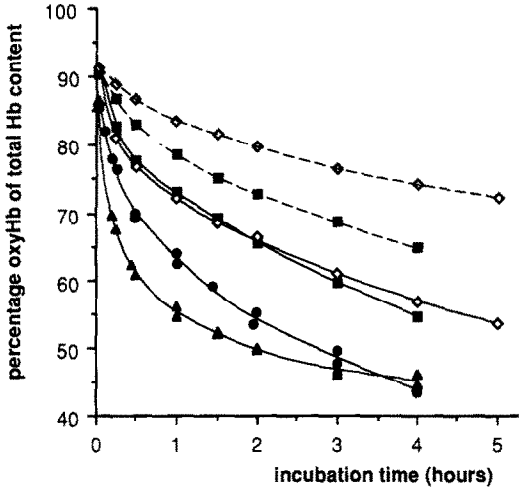

Fig. 2. Effect of reducing substances on autoxidation of oxyHb with or without CPZ. OxyHb (0.1 mM) was incubated in phosphate buffer pH 6.5 (ionic strength 0.1 M) in the dark at 37° in the presence of 2.0 mM CPZ (solid lines) or without CPZ (broken lines). No added reducing substances (◇); 1.0 mM NADPH (■), cytochrome *c* reductase (0.1 unit/ml) had no effect on this NADPH induced autoxidation; 1.0 mM NADPH + 10 μM methylene blue (●); 1.0 mM ascorbic acid (▲). In these last two incubation mixtures the curves with and without CPZ are practically identical.

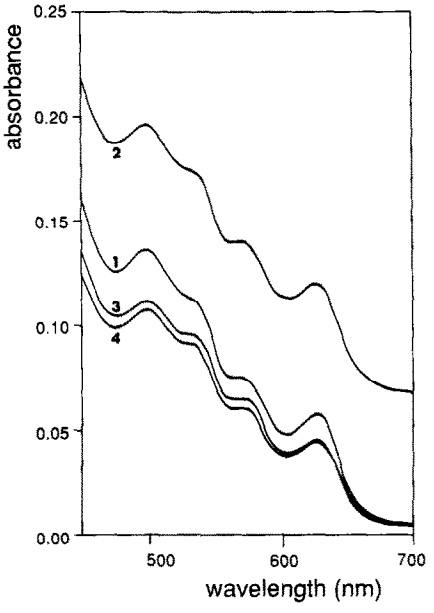

Fig. 3. Visible absorption spectra of metHb during the incubation with CPZ. MetHb (0.1 mM) was incubated in phosphate buffer pH 6.5 (ionic strength 0.1 M) in the dark at 37°, with 2.0 mM CPZ. Spectra were recorded after 30-fold dilution at time intervals: 2 min (1); 1 hr (2); 3 hr (3); 5 hr (4). At the start of the experiment, metHb contained 7% oxyHb.

incubation with purified metHb, the spectrum only changed in the presence of CPZ (Fig. 3). Commercial metHb showed comparable spectral changes both in the presence and absence of CPZ. The metHb spectral changes were caused by a turbidity developed during incubation. As a consequence of the turbidity and precipitation in the presence of CPZ, an absorbance increase over the entire wavelength range was observed, on which either the oxyHb or metHb

spectrum was superimposed (Figs 1B and 3; curves 2). After approximately 2 hr the solutions became clear (due to complete precipitation) and the final concentrations of oxyHb and metHb were 95% and 80% of the initial concentrations, respectively. The decrease in CPZ concentrations was equal to the decrease in oxyHb (5%) or metHb concentration (20%). Because CPZ was added to oxyHb as well as metHb in a molar ratio of 20:1, it can be concluded that 20 CPZ molecules precipitate with 1 oxyHb or metHb molecule. The purified Hb solutions without CPZ stayed clear, whereas the commercial Hb solutions gave turbidities also in the absence of CPZ. Further experiments were done with purified Hb.

Incubations in monooxygenase mimicking systems

We have studied several systems with Hb which have been reported to be useful for mimicking monooxygenase activity with Hb (see introduction). Next to 0.1 mM purified oxyHb or metHb and 2 mM CPZ the incubation mixtures contained: (1) 1 mM NADPH; (2) 1 mM NADPH with 10 μM methylene blue; (3) 1 mm NADPH with 0.1 unit/ml cytochrome *c* reductase; (4) 1 mM ascorbic acid.

CPZSO and other CPZ metabolites were not observed in the mixtures (1) to (3) after 4 hr of incubation. The CPZ concentration decreased in these mixtures to the same extent as reported for the incubations with only oxyHb or metHb (5% and 20%, respectively). The mixtures containing ascorbic acid (mixture 4) showed slight formation of CPZSO: After 4 hr a CPZSO concentration of 0.15 mM (7%) was reached in the mixtures with oxyHb as well as metHb, the CPZ concentration was diminished with 0.34 mM (17%) and 0.62 mM (31%), respectively. The control incubation without Hb did not show CPZSO formation.

The absorption spectra recorded during the incubation of oxyHb in the above mentioned mixtures all revealed a decrease in oxyHb and formation of metHb. The extent of this oxidation of oxyHb was different for each of the mixtures (1) to (4) (Fig. 2). NADPH, whether with or without cytochrome *c* reductase gave the smallest, whereas ascorbic acid and NADPH plus methylene blue gave the largest acceleration of the oxyHb oxidation. Only in the case of NADPH (with and without cytochrome *c* reductase) CPZ showed an additional effect on the oxidation of oxyHb, but the overall effect was not larger than the autoxidation caused by CPZ alone.

The absorption spectra recorded during the incubations of metHb in the mixture (1) to (4) all showed an initial conversion of metHb into oxyHb, followed by a reversed reaction to metHb (Fig. 4). The highest reduction rate was observed in the mixture with NADPH plus methylene blue, followed by ascorbic acid. The mixtures with merely NADPH and with NADPH plus cytochrome *c* reductase only gave a small reduction. The control mixtures without CPZ showed approximately the same absorbance changes (Fig. 4), which implies that the effect of CPZ on the reduction of metHb in these monooxygenase mimicking systems is only minimal.

Incubations with hemoglobin in the presence of H_2O_2

OxyHb and metHb in the presence of H_2O_2 did convert CPZ into CPZSO (Table 1). All reactions mentioned in Table 1 are completed within 5 min. The yield of CPZSO in these incubations was dependent on whether oxyHb or metHb was used. MetHb gave in all cases a higher CPZSO yield. Besides CPZSO the HPLC analysis revealed one other (minor) product with a retention time of 3.0 min. This metabolite showed an absorption spectrum comparable with that of CPZSO. The amount of

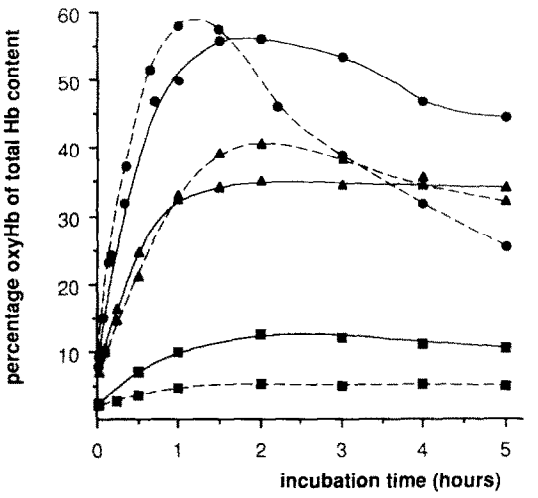

Fig. 4. Effect of reducing substances on the conversion of metHb into oxyHb with or without CPZ. MetHb (0.1 mM) was incubated in phosphate buffer pH 6.5 (ionic strength 0.1 M) in the dark at 37° in the presence of 2.0 mM CPZ (solid lines) or without CPZ (broken lines). Added reducing substances: 1.0 mM NADPH (■), cytochrome *c* reductase gave only a slight increase of these curves; 1.0 mM NADPH + 10 μM methylene blue (●); 1.0 mM ascorbic acid (▲). MetHb without additives was not converted into oxyHb.

Table 1. Conversion of CPZ into CPZSO by hemoglobin with H_2O_2*

Reaction mixtures CPZ (mM)		Percentage recovery CPZ	CPZSO
2.0	oxyHb	72.5	14.5
	metHb	64.0	23.0
	Fe^{2+}†	79.9 (97.5‡)	0.0
	Fe^{3+}†	89.2 (99.0‡)	0.0
	—	100	0.0
	—	100	0.0
1.0	oxyHb	58.0	24.0
	metHb	29.0	42.0
0.50	oxyHb	19.4	45.0
	metHb	0.0	52.0
0.050	oxyHb§	50.0	22.4
	metHb§	0.0	55.0

* Various concentrations of CPZ were incubated for 5 min with oxyHb or metHb (0.1 mM(and H_2O_2 (ten-fold concentration relative to hemoglobin) in phosphate buffer pH 6.5 (ionic strength 0.1 M) plus 0.2 mM EDTA in the dark at 37°.

† 0.4 mM.

‡ CPZ yield in buffer without EDTA.

§ 10 μM.

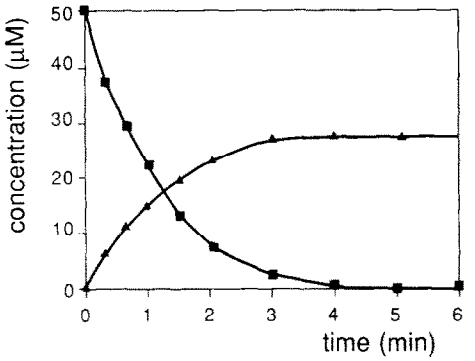

Fig. 5. Conversion of CPZ into CPZSO by metHb with H_2O_2. Fifty μM CPZ was incubated with 10 μM metHb in phosphate buffer pH 6.5 (ionic strength 0.1 M) in the dark at 37°. The reaction was started by adding 0.1 mM H_2O_2. Analyses were performed with HPLC. CPZ (■); CPZSO (▲).

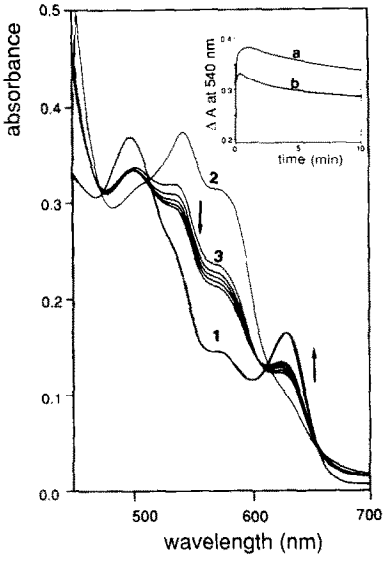

Fig. 6. Changes in the metHb spectrum induced by H_2O_2 and CPZ. (1) MetHb (10 μM) was incubated in phosphate buffer pH 6.5 at 37°; (2) in the presence of 0.1 mM H_2O_2 (1 min after adding H_2O_2: spectrum of ferrylHb); (3) in the presence of 0.1 mM H_2O_2 and 50 μM CPZ (1, 2, 3, 4 and 5 min after adding H_2O_2; arrows indicate the direction of the absorbance change with time). Inset: time course of metHb oxidation by H_2O_2 without (a) and with (b) CPZ followed at 540 nm.

this metabolite was maximum under the last two conditions in Table 1, but was still less than 2% of the initial amount of CPZ (based on equal molar absorptivities of this product and CPZSO).

Control mixtures with omission of oxyHb or metHb did not show CPZSO or other product formation. With 0.4 mM of ferro or ferri ions (corresponding with 0.1 mM oxyHb or metHb, respectively) also no CPZSO formation was found. However, in the presence of H_2O_2 the CPZ concentration was decreased after 5 min (Table 1). In the HPLC chromatograms product peaks, besides deformation of the solvent peak, were not observed. When EDTA was omitted the CPZ yield was nearly 100%. When, instead of CPZ, CPZSO was incubated with metHb or oxyHb and H_2O_2 under the same conditions as in Table 1, the concentration of CPZSO did not change and the formation of other products was not observed.

Figure 5 shows the time course of the conversion of 50 μM CPZ into CPZSO during the incubation with 10 μM metHb and 0.1 mM H_2O_2. During this reaction the amount of CPZSO formed accounted only for 55% of the amount of CPZ disappreared. Under the conditions used here no precipitation, as described before, occurred. Absorption spectra recorded during this reaction were compared with the metHb spectrum and the spectrum of ferrylhemoglobin (ferrylHb) [16], observed 1 min after the addition of H_2O_2 to metHb (Fig. 6). Reactions of metHb with H_2O_2 were followed spectrophotometrically at 540 nm, which is a maximum of absorption of the ferryl species. The results showed (Fig. 6, inset) that in the presence of CPZ the maximum was reached earlier (after 0.3 min instead of 1.0 min) and that the absorbance increase was smaller than without CPZ. From both the spectra and the absorbance time courses, it is apparent that during the reaction with CPZ, metHb is transformed only partly into ferrylHb. This could be due to a quick reaction of CPZ with ferrylHb transforming it back into metHb.

DISCUSSION

Incubation of CPZ with purified or commercially available oxyHb or metHb does not transform CPZ. This finding contradicts the assumption of Minder *et al.* [7] and Traficante *et al.* [8] who reported considerable sulfoxidation of CPZ in whole blood and who assumed that Hb catalyses the sulfoxidation.

Our results are in agreement with those of Kaul *et al.* [9]. However, these authors used commercial Hb. It is possible that commercial Hb contains enzymes like superoxide dismutase, catalase and glutathion peroxidase. These enzymes are likely to have an influence on oxidation reactions catalysed by hemoproteins. We, therefore, performed our experiments also with a purified Hb, which was explicitly checked on the absence of above mentioned enzymes. Commercial Hb contains about 70% metHb and although Kaul *et al.* [8] did not differentiate in their experiments between oxyHb and metHb, we can now report that both oxyHb and metHb do no catalyse the sulfoxidation of CPZ.

The turbidity developed during the incubations of oxyHb and metHb with CPZ is caused by the formation of solid complexes of CPZ with hemoglobin in phosphate buffer as reported by Carlotti *et al.* [24]. Phenothiazine drugs form ion-pairs with phosphates and these more lipophilic ion-pair anions bind to Hb. They reported a ratio of 20 molecules CPZ bound to one molecule of bovine Hb. Actually this is the ratio we also found for purified human Hb.

Although CPZ is not transformed by oxyHb or metHb, CPZ does enhance the autoxidation of oxyHb. The accelerated oxidation of oxyHb (in human erythrocyte suspensions and hemolysates) by phenothiazines was also observed by Okano *et al.* [25].

They presume that this oxidation is caused by phenothiazine radical cations, first produced by some oxidizing substances in the incubation mixtures. CPZ and other phenothiazines are excellent electron-donating drugs, which upon oxidation form relatively stable radical cations. These radical cations are intermediates in the sulfoxidation [26]. Since we do not observe any CPZSO formation and the fact that we used purified Hb without oxidizing substances, the mechanism proposed by Okano *et al.* [25] is unlikely in our incubations.

A possible mechanism for the substrate stimulated autoxidation of oxyHb is that suggested by Mieyal and Blumer [27], in the case of aniline. These authors presume that aniline interacts with Hb at a site other than the heme-iron. This may sufficiently distort the iron oxygen bond so that electron transport from iron (II) to oxygen would be facilitated, and thus the formation of metHb would be stimulated. This mechanism could be applicable for the CPZ enhanced autoxidation.

A direct electron transfer from CPZ to heme bound oxygen, which is the presumed mechanism for the oxidation of oxyHb by reducing substances (ascorbic acid, NADPH plus methylene blue [12, 13, 28]) is excluded because CPZSO would have been produced, via the CPZ radical cation, and this is not observed. A direct electron transfer from CPZ to heme-iron in metHb is also excluded because CPZ does not reduce metHb to oxyHb, as do ascorbic acid and NADPH plus methylene blue. NADPH itself (and in the presence of cytochrome *c* reductase) is a poor donator of electrons to either heme bound oxygen and heme-iron [11, 12].

In both processes, oxyHb oxidation and metHb reduction by ascorbic acid or NADPH plus methylene blue, it is reported [12, 13] that H_2O_2 is produced. H_2O_2 can oxidize oxyHb and metHb further to a compound I or compound II-like form [16, 17] which is capable of oxidizing substrates, as will be discussed below.

Only in the incubations with ascorbic acid some CPZSO was obtained. Probably only with ascorbic acid such an amount of H_2O_2 is produced to form some of the reactive Hb form, which consequently oxidizes CPZ. In the incubation with NADPH and cytochrome *c* reductase Mieyal *et al.* [11] could not detect free H_2O_2.

Reaction with H_2O_2 transforms oxyHb and metHb into a higher oxidation state and thus forms the so-called ferrylHb [16, 17]. The formal oxidation state of ferrylHb is IV (comparable with compound II) although two redox equivalents of H_2O_2 are consumed per metHb-monomer. The other redox equivalent is located ultimately at the apoprotein as an amino acid residue radical. Whether this amino acid radical is formed directly by H_2O_2 or via short-lived intermediates like compound I or hydroxyl radicals (produced in a homolytic cleavage of H_2O_2) is not known [17]. CPZ, when present, obviously reacts with one or more of these reactive Hb forms under formation of CPZSO. In this respect the reaction of CPZ with metHb in the presence of H_2O_2 could be compared with that of CPZ with horseradish peroxidase and H_2O_2 [29]. In this reaction CPZ reacts with both compound I and compound II and is transformed to the CPZ radical cation. This CPZ radical cation decays, catalysed by buffer ions, to CPZ and CPZSO (50% of each in phosphate buffer) [30]. Under non-limiting H_2O_2 conditions all CPZ is converted by Hb. However only 55% is transformed into CPZSO instead of nearly 100% as would be expected because only one minor metabolite (accounting for less than 2% of CPZ) was observed. We assume that, like in the case of horseradish peroxidase, the CPZ radical cation is produced as an intermediate and that a part of these radicals covalently bind to the Hb apoprotein. The CPZ radical cation has been shown to bind covalently to biological macromolecules like albumin, RNA and DNA [31].

Under H_2O_2 limiting conditions oxyHb produces less CPZSO compared with metHb: in the case of oxyHb more H_2O_2 equivalents are needed to form the reactive Hb form, so less H_2O_2 is left for the conversion of CPZ. CPZSO is not converted by the peroxidase-like activity of Hb.

Hb is often claimed to be a Fenton catalyst but this has never been established [32]. The sulfoxidation of CPZ by Hb and H_2O_2 is not based on a Fenton-type reaction because ferro and ferri ions together with H_2O_2 do not produce CPZSO. The loss of CPZ under these conditions (Table 1) could be due to the formation of other metabolites (observed by HPLC as a deformation of the solvent peak) in a Fenton-type reaction. This loss is observed only in the EDTA containing buffers which could be explained by the fact that the iron–EDTA complex is a far better Fenton catalyst than unliganded iron [32].

Concluding, our study demonstrates that Hb catalyses the sulfoxidation of CPZ only from a higher oxidation state. This could be a form like compound I or compound II (ferrylHb). This is an interesting finding in view of the similarity with the active form of cytochrome P-450 [17]. *In vivo* one might expect that only under conditions where a considerable amount of H_2O_2 is produced in the erythrocyte, as might be the case with sickle cell anaemia [33], Hb can act as a peroxidase thus metabolizing some drugs, for instance CPZ.

Due to the questions raised the reaction of CPZ with Hb and H_2O_2 will be further investigated.

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