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MECHANISM OF INACTIVATION OF THYROID PEROXIDASE BY THIOUREYLENE DRUGS

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Abstract—We have investigated the mechanism by which the thioureylene drugs, 1-methyl-2-mercaptoimidazole (MMI) and 6-*n*-propylthiouracil (PTU), inactivate thyroid peroxidase (TPO). Our results indicate that inactivation of TPO by MMI and PTU involves a reaction between the drugs and the oxidized heme group produced by interaction between TPO and H_2O_2. This conclusion is supported by the following observations. First, addition of a low concentration of H_2O_2 to a solution of TPO shifted λ_{max} of the Soret band from 411 to 420 nm, reflecting the formation of an oxidized form of TPO (TPO_{ox}). Addition of MMI or PTU to TPO_{ox} produced a Soret spectrum that was significantly different from the spectrum of native TPO or TPO_{ox}, whereas addition of MMI or PTU to native TPO produced no significant change in the heme spectrum. Second, studies with radiolabeled MMI and PTU combined with simultaneous assays of enzyme activity (guaiacol assay) showed that firm binding of the drugs to TPO and inactivation of the enzyme occurred on addition of the drugs to TPO_{ox}. However, neither binding nor inactivation occurred on addition of the drugs to native TPO. Third, the presence of a low concentration of iodide prevented the shift in the Soret spectrum, the binding of labeled drug, and the loss of enzyme activity associated with the addition of thioureylene drugs to TPO + H_2O_2. Under these conditions we assume that the enzyme was present as $TPO \cdot I_{ox}$, a form in which the heme is present in the same reduced state as in native TPO. This would explain the protective action of iodide on the inactivation of TPO_{ox} by MMI and PTU.

Various groups of investigators have studied the mechanism of action of the thioureylene antithyroid drugs [1–8]. It is well accepted that these drugs inhibit thyroid peroxidase (TPO)-catalyzed iodination of thyroglobulin.

In a previous communication [3] we reported that the inhibition of TPO-catalyzed iodination by 1-methyl-2-mercaptoimidazole (MMI) and 6-*n*-propylthiouracil (PTU) may be reversible or irreversible, depending on the iodide to drug concentration ratio. Subsequently, it was shown by Davidson *et al.* [5] that thioureylene drugs, especially MMI, could irreversibly inactivate TPO in the presence of H_2O_2 and that this inactivation could be prevented by iodide. This observation suggested a mechanism for the irreversible inhibition of TPO-catalyzed iodination that we had described previously. Further support for such a mechanism was provided recently by Edelhoch *et al.* [8], who studied the effect of thioureylene compounds on lactoperoxidase (LPO) activity in the presence and absence of iodide. They reported that thioureylene drugs could rapidly inactivate LPO and that the rate of this inactivation was markedly inhibited by iodide.

In the present paper, we describe our studies on the mechanism by which thioureylene drugs inactivate TPO. We have confirmed and extended the results of Davidson *et al.* [5], employing a much more highly purified TPO preparation than that used in their studies. The availability of highly purified TPO enabled us to use the Soret spectrum of TPO to follow effects of added thioureylene drugs and iodide. We were also able to examine the binding of radiolabeled MMI and PTU to TPO and to correlate this with effects on the Soret spectrum and on enzyme activity. Our results support the view that inactivation of TPO by MMI and PTU involves a reaction between the drug and the oxidized heme group in the peroxidase.

METHODS AND MATERIALS

The effect of thioureylene drugs on TPO was followed by three different procedures: (1) spectrophotometric recording of the Soret band of TPO, (2) use of radiolabeled PTU and MMI to study binding of the drugs to TPO, and (3) assay of TPO enzyme activity by catalysis of guaiacol oxidation. Generally, all of these measurements were made using the same initial incubation mixture. The TPO concentration was generally 20 μg/ml, and the incubation medium was 0.067 M phosphate (pH 7.0). Based on a molecular weight of 90,000 [9] and an assumed purity of 80% (see below), the TPO concentration was 0.18 μM.

Spectrophotometric studies. A Cary 219 spectrophotometer equipped with a wave length programmer and timer was used for measurement of the Soret spectrum. This instrument is admirably suited for accurately recording repetitive scans on the same solution. One milliliter cuvettes were used to conserve enzyme. Additions of various agents (H_2O_2, [^{14}C]MMI, [^{35}S]PTU, I^-) were made in 2 μl of solution with a special cuvette mixer.

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Binding of radiolabeled drugs. [^{14}C]MMI (32 mCi/mmole, labeled in the 2 position) and [^{35}S]PTU (113 mCi/mmole) were purchased from Amersham (Arlington Heights, IL). The labeled drugs were dissolved in 0.067 M phosphate–0.1 mM EDTA (pH 7.0) and added to the TPO solution in the cuvette to determine their effect on the Soret spectrum. After the scans had been recorded and small aliquots removed for assay of enzyme activity (see below), the incubation mixture was dialyzed against a large volume of 0.067 M phosphate (pH 7.0), with one or two changes of buffer. The fraction of the initial radioactivity remaining in the dialysis bag was determined by liquid scintillation counting, and moles of drug bound per mole of enzyme was calculated from the initial drug concentration, the fraction of the radioactivity remaining in the dialysis bag, and the estimated concentration of TPO.

Guaiacol assay. Ten microliters of the incubation mixture (0.2 μg TPO) was transferred to a 3 ml cuvette containing 2.1 ml of 33 mM guaiacol in 0.067 M phosphate (pH 7.0). Guaiacol oxidation was initiated by addition of 10 μl of 66 mM H_2O_2, and ΔA_{470} was recorded with a model 124 Hitachi double beam spectrophotometer connected to a Sargent–Welch model SRG recorder. The absorbance change in 1 min was used as a measure of enzyme activity. With untreated TPO, the value for ΔA_{470} averaged 0.42 after subtraction of a small blank. Enzyme activity after the addition of various agents was expressed as the percentage of that observed in the untreated TPO solution.

Thyroid peroxidase. The experiments reported in the present study were performed with Preparation X, purified from frozen hog thyroid glands essentially as described for Preparation VII in a previous communication from this laboratory [9]. Preparation X displayed a value for A_{410}/A_{280} of 0.50, compared to 0.54 for Preparation VII. Tests for purity were not performed with Preparation X. However, based on its value for A_{410}/A_{280} and on its specific activity in an iodination assay, we estimate that it was about 90% as pure as Preparation VII. Since the latter was shown to be about 90% pure [9], we estimate that Preparation X was about 80% pure.

RESULTS

Inactivation of TPO by MMI in the presence and absence of H_2O_2; protective effect of iodide. Figure 1 shows the effect on TPO activity of treatment of a solution containing 5 μg/ml TPO with various concentrations of MMI or PTU for 1 min in the presence and absence of H_2O_2 and I^-. In the presence of 125 nM H_2O_2, TPO was completely inactivated by 3 μM MMI and 50% inactivated by 0.9 μM MMI. PTU also inactivated TPO under the same conditions, but it was less potent than MMI. The presence of 100 μM I^- completely protected TPO against the inactivating effect of both drugs. These results are similar to those previously reported by Davidson *et al.* [5]. Exposure of TPO to thioureylene drugs in the absence of H_2O_2 also resulted in significant inactivation of the enzyme, as we have reported previously [10]. However, the inactivating effect of the drugs was much greater in the presence of H_2O_2, indicating that the oxidized form of the TPO (TPO_{ox}) is more sensitive than the native* form to the inactivating effect of thioureylene drugs. Iodide protected both forms against inactivation. Treatment of 5 μg/ml TPO with 125 nM H_2O_2 alone for 1 min produced no inactivation of the enzyme (results not shown). In the absence of H_2O_2, the inactivation of TPO by MMI and PTU was rather inconsistent and appeared to be largely dependent on the concentration of TPO. When the TPO concentration was as low as 1 μg/ml, the inactivation was consistently observed, whereas at 20 μg/ml TPO (see below) little or no inactivation was detected. At 5 μg/ml TPO, inactivation by 10 μM MMI was observed in some experiments (as in Fig. 1) but not in others.

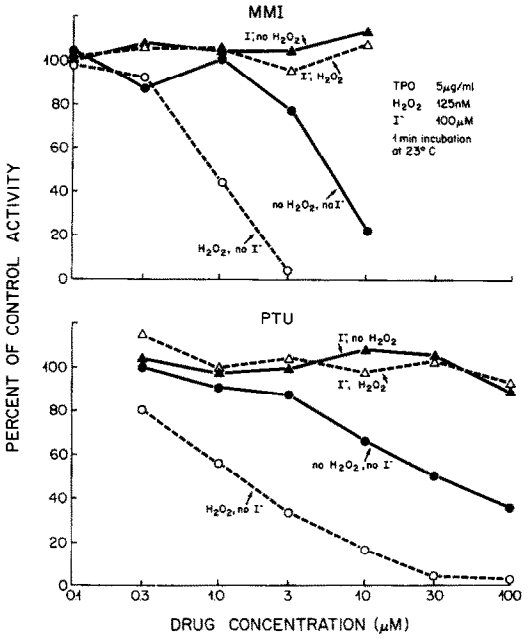

Fig. 1. Inactivation of TPO by MMI and PTU in the presence and absence of H_2O_2; protective effect of iodide.

Soret spectrum of TPO; effects of H_2O_2, MMI, and iodide. Figure 2A shows the Soret spectrum of TPO before and after addition of H_2O_2. Native TPO showed a well defined peak with λ_{max} at 411 nm. Addition of 2 μM H_2O_2 shifted λ_{max} to 420 nm, reflecting the formation of TPO_{ox}. Based on the mechanism of horseradish peroxidase (HPR)-catalyzed oxidation proposed by Chance [11] and by George [12, 13], it seems likely that TPO_{ox} is analogous to either Compound I or Compound II of horseradish peroxidase. Since Ohtaki *et al.* [14, 15] reported that Compound I of TPO is very unstable and is converted very rapidly and spontaneously to Compound II, we assume that the spectrum of TPO_{ox} in Fig. 2A represents that of Compound II.

Figure 2B shows the effect of adding 2 μM MMI to TPO_{ox}. Addition of the drug produced a shift in λ_{max} of the Soret band from 420 to 414–415 nm. A

* The "native form" of TPO as used in the present paper refers to the TPO isolated in our purification procedure. We do not imply that it represents the native form of the enzyme as it exists *in vivo*.

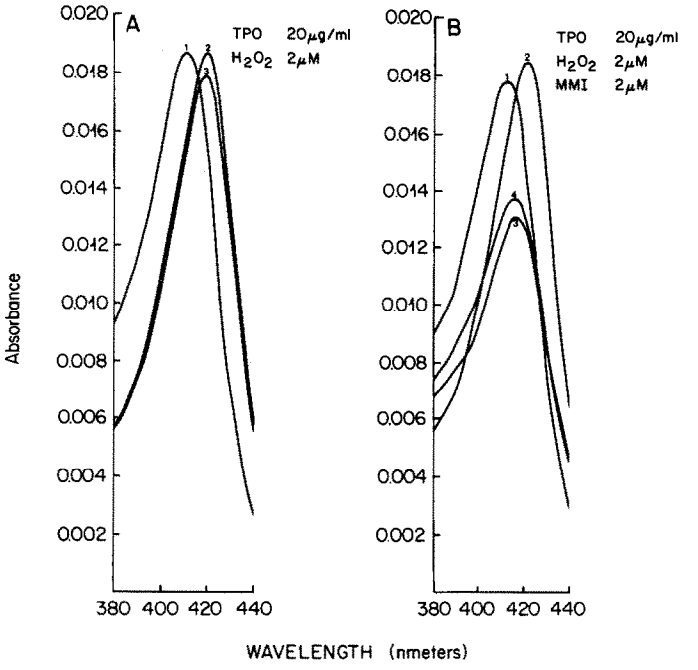

Fig. 2. Soret spectrum of TPO under varying conditions. (A) Curve 1, native TPO; curve 2, scan started immediately after addition of H_2O_2; and curve 3, repeat scan started 4 min after H_2O_2. (B) Curve 1, native TPO; curve 2, scan started immediately after addition of H_2O_2; curve 3, MMI added immediately after completion of scan with H_2O_2; and curve 4, repeat scan started 4 min after addition of MMI. Spectrophotometer settings: full scale absorbance = 0.02, scanning rate = 1 nm/sec.

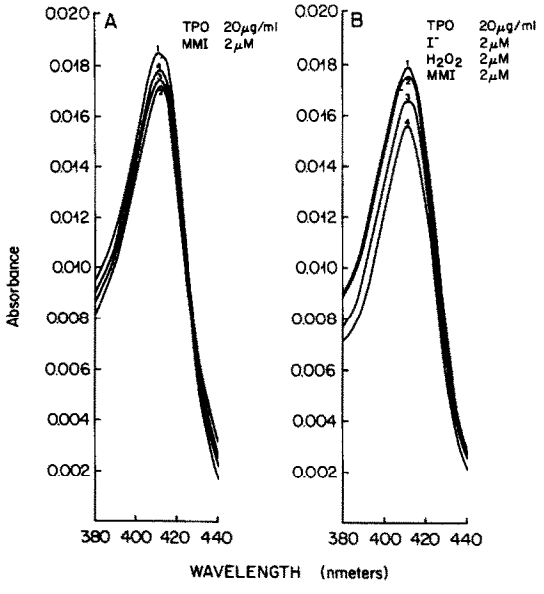

Fig. 3. Soret spectrum of TPO under varying conditions. (A) Curve 1, native TPO; curve 2, scan started immediately after addition of MMI; curve 3, repeat scan started 4 min after MMI; and curve 4, repeat scan started 8 min after MMI. (B) Curve 1, native TPO; curve 2, scan started immediately after addition of I^-; curve 3, H_2O_2 added immediately after completion of scan with added I^-; and curve 4, MMI added immediately after completion of scan with added H_2O_2. The progressive decrease in absorbance at λ_{max} observed in curves 1–4 was at least partially attributable to dilution by successive addition of reagents. Spectrophotometer settings were as in Fig. 1.

decrease in absorbance was also evident. These observations suggested that the drug reacted with the oxidized heme group in TPO_{ox} to form a new molecular species. As indicated below, enzyme activity was completely abolished and MMI appeared to be covalently linked to the TPO.

Figure 3A shows that addition of 2 μM MMI to TPO in the absence of H_2O_2 had no effect on λ_{max} of the Soret band. Moreover, under these conditions there was no effect of MMI on enzyme activity. We conclude from these results that MMI reacts only with the oxidized form of the heme in TPO and that this reaction results in loss of enzyme activity.

Figure 3B shows the effect of I^- on the spectral shifts produced by H_2O_2 and by MMI on the Soret band of TPO. In the presence of 2 μM I^-, addition of H_2O_2 did not produce the characteristic shift in the Soret band shown in Fig. 2. Addition of MMI to TPO following addition of I^- and H_2O_2 also produced no spectral shift in λ_{max} of the Soret band (Fig. 3B). This accords with the results in Fig. 3A, which show that MMI did not react with the reduced form of the heme. The presence of iodide not only prevented the spectral changes seen after addition of MMI to TPO_{ox} but it also prevented the loss of enzyme activity and the binding of [^{14}C]MMI to TPO, as described in the following section.

Correlation between binding of [^{14}C]*MMI to TPO and enzyme inactivation; protective effect of iodide.* Binding of [^{14}C]MMI and enzyme activity were measured simultaneously on the samples that were used to obtain the spectra shown in Figs. 2 and 3. The results are presented in Table 1. Addition of 2 μM

Table 1. Correlation between binding of [^{14}C]1-methyl-2-mercaptoimidazole to thyroid peroxidase and enzyme inactivation; effect of iodide

TPO concn (μg/ml)	Estimated TPO concn (μM)	I^- (μM)	H_2O_2 (μM)	MMI (μM)	Enzyme activity (% of initial)	Moles [^{14}C]MMI bound per mole of TPO
20	0.18	0	0	2	98	0.045
20	0.18	0	2	2	0.4	1.08
20	0.18	0.2	2	2	74	0.17
20	0.18	2	2	2	95	0.068
20	0.18	20	2	2	90	0.082
20	0.18	0	2	0	70	
20	0.18	2	2	0	99	

[^{14}C]MMI to a solution containing 20 μg/ml TPO had no significant effect on enzyme activity and resulted in little or no binding of ^{14}C to the TPO. Under the same conditions, the drug also had no effect on the Soret spectrum of TPO (Fig. 3A). However, if the TPO was first converted to TPO_{ox} by addition of H_2O_2, then addition of 2 μM MMI resulted in complete elimination of enzyme activity, and the amount of ^{14}C that was firmly bound corresponded closely to 1 molecule of [^{14}C]MMI per molecule of enzyme. Under these conditions, the spectral data (Fig. 2B) indicate the formation of a new molecular species, which we believe to be the product of a reaction between MMI and the oxidized heme in TPO_{ox}.

All of the above described effects of MMI on TPO were prevented by I^-. As shown in Table 1, the presence of only 2 μM I^- prevented both the inactivation of the enzyme by 2 μM MMI and the binding of ^{14}C to the enzyme. Partial inhibition was achieved with only 0.2 μM I^-. Figure 3B shows that 2 μM I^- also prevented the shifts in the Soret band of TPO induced by H_2O_2 and by MMI.

Results obtained with PTU. Experiments analogous to those described above for MMI were also performed with PTU. The spectral results shown in Figs. 2 and 3 were reproduced with PTU, the only difference being that 4 μM PTU was used compared to 2 μM MMI (results not shown). Effects of 4 μM PTU on binding and on enzyme inactivation are shown in Table 2. As in the case of MMI, PTU did not inactivate TPO in the absence of H_2O_2. In the presence of H_2O_2, the inactivation was very marked but not as complete as that produced by 2 μM MMI. Although there was a correlation between binding and inactivation, the quantitative relationship between the two was not as clear as in the case of MMI, as shown by the finding that 87% inactivation was associated with the binding of only 0.5 mole of [^{35}S]PTU (or its labeled product) per mole of TPO. We have no simple explanation for this observation. It appears that [^{35}S]PTU may have formed a more dissociable complex with TPO than did [^{14}C]MMI. It would have been of interest to test binding with [^{14}C]PTU and [^{35}S]MMI, but these compounds were not available to us.

Effect of MMI and PTU on TPO-catalyzed guaiacol oxidation. To gain further information about possible mechanisms by which MMI and PTU might inactivate TPO_{ox}, we have studied the inhibitory effects of these drugs on TPO-catalyzed guaiacol oxidation. The results of such a study are illustrated in the Lineweaver–Burk plots shown in Fig. 4. In the absence of drug, a linear plot was obtained, from which a K_m value of 6.7 mM was calculated. In the presence of 10 or 20 μM MMI, there was a marked upward curvature in the Lineweaver–Burk plot. Nevertheless, it was quite evident that the inhibitory effect of 10 or 20 μM MMI on guaiacol oxidation could be completely overcome by raising the guaiacol concentration to 33 mM (the concentration used in the assay procedure). Similar results were obtained with PTU, except that this drug was less potent than MMI as an inhibitor of guaiacol oxidation. The results in Fig. 4 indicate that both MMI and PTU compete with guaiacol for a common site on TPO_{ox}. In the case of guaiacol, binding to this site leads to oxidation of the substrate. However, in the case of MMI and PTU, binding to the same site results in inactivation of the TPO. The upward curvature seen in Fig. 4 for MMI indicates that the rate of oxidation

Table 2. Correlation between binding of [^{35}S]6-*n*-propylthiouracil to thyroid peroxidase and enzyme inactivation; effect of iodide

TPO concn (μg/ml)	Estimated TPO concn (μM)	I^- (μM)	H_2O_2 (μM)	PTU (μM)	Enzyme activity (% of initial)	Moles [^{35}S]PTU bound per mole of TPO
20	0.18	0	0	4	95	0.071
20	0.18	0	2	4	13	0.50
20	0.18	0.2	2	4	61	0.18
20	0.18	2	2	4	86	0.10
20	0.18	20	2	4	87	0.10
20	0.18	0	2	0	78	
20	0.18	2	2	0	87	

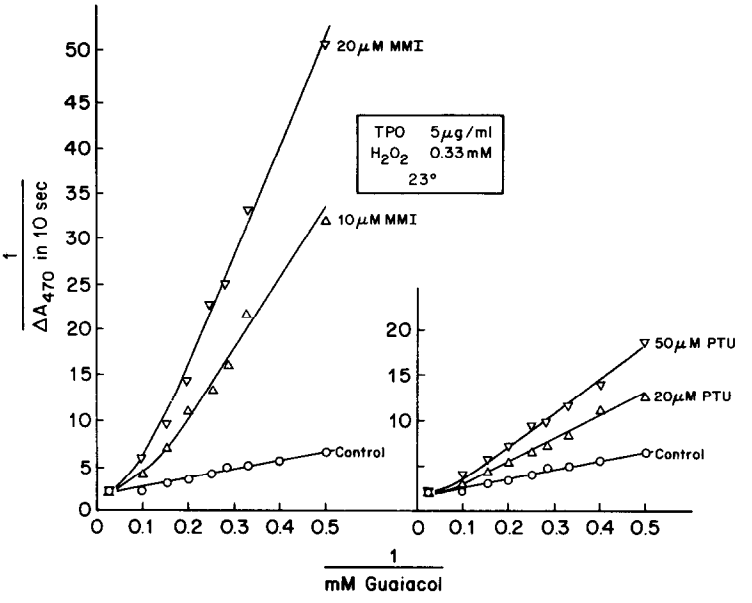

Fig. 4. Lineweaver–Burk plots for inhibition of TPO-catalyzed guaiacol oxidation by MMI and PTU. The incubations were performed directly in the cuvette in 0.067 M phosphate (pH 7.0). The reaction was initiated with 10 μl of 66 mM H_2O_2 added with a Calbiochem plumper. Absorbance was measured with a Gilford spectrophotometer equipped with a digital absorbance meter and a data lister. The instrument was zeroed immediately before addition of H_2O_2. A stopwatch was started simultaneously with the addition of H_2O_2, and absorbance at 10 sec was recorded with the data lister.

of low concentrations of guaiacol in the presence of MMI is less than that expected for simple competitive inhibition. One possible explanation for these observations is that TPO is rapidly inactivated at the lower guaiacol concentrations where guaiacol competes less favorably with MMI.

DISCUSSION

Our preparation of TPO gave a well defined Soret band with λ_{max} at 411 nm. In the presence of H_2O_2, λ_{max} was shifted to 420 nm (Fig. 2), reflecting the formation of oxidized TPO (TPO_{ox}). λ_{max} for TPO_{ox} was stable for at least 10 min, in contrast to the results reported by Ohtaki *et al.* [14]. Their TPO preparation was very impure, and the Soret band disappeared rapidly on the addition of H_2O_2.

As shown in Fig. 2B, the addition of a very low concentration of MMI to TPO_{ox} produced a Soret spectrum that was significantly different from that of TPO_{ox} or native TPO. On the other hand, addition of MMI to native TPO had no effect on the Soret spectrum (Fig. 3A). We conclude from these observations that MMI reacted with the heme in TPO_{ox} to produce a heme product that was different from the heme in TPO_{ox} or in native TPO.

Further evidence for a reaction between TPO and MMI came from studies using [^{14}C]MMI. Under conditions where addition of MMI produced a shift in the Soret spectrum, we observed that an appreciable fraction of the ^{14}C, corresponding to approximately 1 mole of MMI per mole of enzyme, remained bound to TPO after extensive dialysis (Table 1). We interpret this to indicate that either MMI itself or more likely an oxidation product of MMI became covalently linked to the heme. Binding of ^{14}C to the TPO was also correlated with inactivation of the enzyme, suggesting that the reaction between heme and MMI was the cause of enzyme inactivation.

All of the above effects of MMI on TPO were prevented by 2 μM iodide. The effect of I^- on the Soret shift produced by H_2O_2 and by MMI is shown in Fig. 3B. In the presence of 2 μM I^-, the Soret spectrum of native TPO remained unaffected by the addition of H_2O_2. This indicates that the heme was in its reduced rather than in its oxidized form. This is readily explained by assuming that the oxidized heme formed on addition of H_2O_2 was immediately reduced by I^-, with the formation of oxidized iodine (probably I^+). As proposed by Davidson *et al.* [5], the reaction can be written as follows: $TPO_{ox} + I^- \rightarrow TPO \cdot I_{ox}$. In $TPO \cdot I_{ox}$ it may be assumed that the heme exists in its reduced form, and thus the Soret band is indistinguishable from that of native TPO. Addition of MMI to $TPO \cdot I_{ox}$ produced no spectral shift because MMI does not react with the reduced form of the heme. This is consistent with the observation that, in the presence of iodide, MMI did not bind to TPO nor did it abolish enzyme activity.

Michot *et al.* [7] studied the effect of methylthiouracil on the Soret spectrum of the lactoperoxidase–H_2O_2 complex at pH 8.8. They reported a spectral shift on addition of methylthiouracil similar to that seen in Fig. 2B on addition of MMI to TPO_{ox}. This was associated with enzyme inactivation and was prevented by iodide, results similar to those reported in the present manuscript. However, the role of iodide was conceived by Michot *et al.* [7] to be quite different from that proposed by us and by Davidson *et al.* [5]. They concluded that

iodide acts as an enzyme regulator rather than as an oxidizable substrate. We have obtained additional evidence in support of our view that oxidized iodine plays a role in TPO-catalyzed oxidation of thioureylene drugs and this will be presented in a subsequent publication (H. Engler and A. Taurog, manuscript in preparation).

The inhibitory effects of MMI and PTU on TPO-catalyzed guaiacol oxidation were also investigated in the present study. The results, shown in Fig. 4, indicate that these drugs compete with guaiacol for a common site on TPO_{ox}. Guaiacol is a classical donor for peroxidases, and attachment to this site results in catalytic oxidation of the substrate. The thioureylene drugs, on the other hand, are not generally considered to be donors for TPO since they are not catalytically oxidized by TPO + H_2O_2. Nevertheless, our results suggest that, when MMI or PTU attaches to the guaiacol active site in TPO_{ox}, the drugs may also undergo oxidation. However, in this case it appears that the oxidized product remains bound to the heme, and the enzyme, therefore, can no longer function as a catalyst. Davidson *et al.* [5] previously postulated that TPO_{ox} reacts with thioureylene drugs to produce an oxidized drug product that inactivates the enzyme. Our findings provide experimental support for such a mechanism and demonstrate that interaction between the drug and the heme group is reponsible for the enzyme inactivation.

We have shown previously [16] that reduced glutathione (GSH) inhibits TPO-catalyzed iodination. Moreover, Pommier and Cahnmann [17] have shown that GSH can bind to the heme of lactoperoxidase. It was of interest, therefore, to determine whether GSH inactivates TPO. In experiments similar to those shown in Fig. 1, 30 μM GSH was added to a solution containing 5 μg/ml TPO and 125 nM H_2O_2. No significant inactivation of TPO was observed, in contrast to the results obtained with MMI and PTU. The inactivation of TPO by thioureylene drugs, therefore, does not depend on the presence of a sulfhydryl group. This supports the view that PTU and MMI are not true mercaptans [18, 19]. The reaction between TPO_{ox} and true mercaptans such as GSH differs in important respects from that between TPO_{ox} and thioureylene drugs.

In the presence of I^-, as we have shown previously [3], MMI and PTU are oxidized by TPO + H_2O_2. The oxidant in this case is assumed to be $TPO \cdot I_{ox}$. This form of the enzyme, unlike TPO_{ox}, is not inactivated by thioureylene drugs but rather acts to catalyze their oxidation. Moreover, since $TPO \cdot I_{ox}$ is also thought to be the active iodinating agent for thyroglobulin, the competition between thioureylene drugs and tyrosyl residues in thyroglobulin for $TPO \cdot I_{ox}$ provides a basis for the reversible inhibition of iodination that we have described previously [3]. Further studies concerning the mechanism of reversible inhibition will be presented in a subsequent paper (H. Engler and A. Taurog, manuscript in preparation).

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ADDENDUM

After this manuscript had been submitted for publication, we came across the paper by Ohtaki *et al.*[20] dealing with the reactions of TPO with H_2O_2, MMI and tyrosine. In agreement with our findings, they concluded that MMI can inactivate TPO_{ox} (more specifically in their study, Compound II). However, they apparently did not observe the intermediate Soret spectrum for the inactive enzyme that we report in Fig. 2B. Their order of addition of H_2O_2 and MMI was different from ours, but this is unlikely to be the source of the discrepancy, as we were able to observe the intermediate using their order of addition. At present we have no explanation for this discrepancy.