Localization of gastric peroxidase and its inhibition by mercaptomethylimidazole, an inducer of gastric acid secretion

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Mercaptomethylimidazole (MMI) is a potent inducer of gastric acid secretion which is associated with significant inhibition of peroxidase activity of rat gastric mucosa *in vivo*. A time-dependent increase in acid secretion correlates well with time-dependent decrease in the peroxidase activity. In a chamber experiment *in vitro* using isolated gastric mucosa, MMI stimulates acid secretion, showing an almost linear response up to 600 μM . The time-dependent increase in acid secretion is also correlated with time-dependent inhibition of the peroxidase activity. This effect is not mediated through oxidation of MMI by flavin-containing mono-oxygenase, which is absent from gastric mucosa. The peroxidase has been localized mainly in parietal cells isolated and purified from gastric mucosa by controlled digestion with collagenase followed by Percoll-density-gradient centrifugation. Peroxidase activity was further localized in the outer membrane of the purified mitochondria of the parietal cell by some membrane-impermeant reagents, indicating outward orientation of the enzyme. MMI can inhibit the peroxidase activity of both the parietal cell and its mitochondria in a concentration-dependent manner. The possible involvement of the parietal-cell peroxidase- H_2O_2 system in MMI-induced acid secretion may be suggested.

INTRODUCTION

Mercaptomethylimidazole (MMI) is a well-known antithyroid drug of the thionamide group which inhibits thyroid peroxidase-catalysed thyroxine formation [1]. The mechanism of inhibition has been studied by using thyroid peroxidase and the bovine lactoperoxidase- H_2O_2 -iodide system [2,3]. We have previously reported that MMI induces gastric acid secretion in mice, and the effect is partially sensitive to cimetidine, the H_2 -receptor antagonist, and almost completely to omeprazole, the inhibitor of the terminal proton-pumping H^+ - K^+ -ATPase of the parietal cell [4]. We have also reported the presence of a peroxidase in the fundic mucosa of both mouse and rat [5–7]. The enzyme from rat stomach has been purified and characterized as being entirely different from thyroidal and other extra-thyroidal peroxidases [7]. This peroxidase is an endogenous protein of the gastric mucosa, modulated by glucocorticoids [8], and not contributed by the invading eosinophil as is the case in intestine [9]. Glucocorticoids also stimulate acid secretion [10], produce gastric ulcers [11,12] and are potent inhibitors of the gastric peroxidase activity [8]. Non-steroidal anti-inflammatory drugs which stimulate gastric acid secretion and produce gastric ulcers [13–15] also inhibit the gastric peroxidase activity *in vitro* [16]. These studies may reflect the possibility that gastric peroxidase may have some role in the cellular control of acid secretion and gastric ulceration, possibly by controlling the level of intracellular H_2O_2 which may be utilized by the peroxidase to perform some specialized function. In the present paper, we report evidence from experiments both *in vivo* and *in vitro* that MMI-induced acid secretion in rat gastric mucosa correlates well with the inhibition of its peroxidase activity. This necessitates the precise cellular localization of this enzyme in the multicellular gastric mucosa to assign any role of this enzyme in gastric physiology. With this objective, we have attempted to isolate a peroxidase-enriched cell fraction from rat gastric mucosa and to characterize it biochemically, with a view to studying the effect of various acid stimulants on the activity of this enzyme. The present study

indicates that the peroxidase is located in the outer membrane of the mitochondria of the parietal cell, with its catalytic site oriented outside, and is strongly inhibited by MMI, the inducer of acid secretion, suggesting its possible involvement in parietal-cell secretory function.

MATERIALS AND METHODS

Animals

Male Wistar rats (200–250 g body wt.) of our Institute inbred strain were used throughout the experiment.

Reagents

Protease (Pronase E, 4.7 unit/mg), collagenase type I (295 units/mg), trypsin inhibitor, trypsin, diaminobenzidine, *p*-chloromercuribenzenesulphonic acid (PCMBS), histamine, carbachol, isobutylmethylxanthine, BSA, EGTA and MMI were obtained from Sigma, St. Louis, MO, U.S.A. Na^{125}I was obtained from the Bhabha Atomic Research Centre, Trombay, India. Dimethyl[^{14}C]aminopyrine (96 mCi/mmol) and Percoll were obtained from Flurochem, U.K., and Pharmacia, Uppsala, Sweden respectively. Hanks' balanced salt solution (HBSS) was prepared in accordance with instructions given in the GIBCO manual. Cetyltrimethylammonium bromide was obtained from Sisco Research Laboratories, Bombay, India, and all other chemicals were of analytical-reagent grade.

Isolation of dispersed mucosal cells

Cells were isolated by modifications of previously described methods [17–19]. The pinkish fundic portion of the stomach of three non-fasted rats was rinsed in chilled HBSS, pH 7.4, minced quickly and transferred to a double-layered glass chamber (for water circulation at constant temperature) containing 20 ml of HBSS with 1 mg of protease/ml. The tissue was digested at 37 °C with continuous gassing with O_2/CO_2 (19:1) and magnetic stirring for 15 min. The released cells were decanted through a coarse nylon mesh and centrifuged at 4000 rev./min for 1 min.

Abbreviations used: MMI, mercaptomethylimidazole; PCMBS, *p*-chloromercuribenzenesulphonic acid; DAS, diazonium salt of sulphanilic acid; HBSS, Hanks' balanced salt solution; AP, aminopyrine.

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Fig. 1. Schematic diagrams of (a) the separated cells of the F_2 fraction after discontinuous Percoll-gradient centrifugation and (b) the discontinuous-sucrose-gradient centrifugation of the 12000 g pellet of parietal-cell homogenate

(a) The whole process of centrifugation, including the preparation of the cells, the gradient and the number of cells loaded, was described in the Materials and methods section. (b) Centrifugation was done in 5 ml transparent tubes after loading 0.5 ml of organelles on a 4.5 ml gradient.

The resulting cell pellet was washed with HBSS and termed the F_1 fraction. The remaining tissue was digested for another 60 min at 37 °C in 20 ml of HBSS containing 0.1 % BSA, 1 mM-EGTA, 0.2 mg of trypsin inhibitor/ml and 0.5 mg of collagenase/ml as above. The cell suspension was passed through the nylon mesh, washed three times in HBSS and suspended in oxygenated HBSS containing 0.1 % BSA but without Ca^{2+} . This cell fraction was termed the F_2 fraction. Next, 10^7 cells of the F_2 fraction were suspended in 1.5 ml of HBSS [saturated with O_2/CO_2 (19:1)] without Ca^{2+} but with BSA (0.1 %) and layered over a discontinuous gradient comprising 3 ml each of 10 %, 20 % and 30 % (v/v) Percoll in HBSS [19] in a Du Pont 15 ml centrifuge tube. The gradients were run at 1075 g for 10 min at room temperature in a Sorvall RC5B centrifuge. The mixed cell population was resolved into four bands, B_1 , B_2 , B_3 and B_4 , as shown in Fig. 1(a). Cells were collected, washed three times in HBSS to remove adhering Percoll and finally suspended in HBSS for further study.

Measurement of accumulation of [^{14}C]aminopyrine ([^{14}C]AP)

This was done by the method described previously [17,19]. Cells were kept at room temperature for 30 min after isolation. Some 3×10^5 cells were added to 10 ml plastic scintillation vials containing 1 ml of HBSS with Ca^{2+} (1 mM), 0.1 % BSA, pH 7.4, and 0.1 μ Ci of [^{14}C]AP (96 mCi/mmol) in the absence or presence of the test drug. Cells were incubated for 30 min at 37 °C with continuous shaking (120 rev./min) and aeration. Samples (0.9 ml) were transferred to Microfuge tubes, and cells were pelleted by centrifugation (15000 g -min). The supernatant was decanted and the inside of the tube was carefully blotted dry. Then 0.3 ml of 3 M-KOH was added to the cell pellet and kept for 2 h with occasional vortex-mixing. From this 0.2 ml was added to 5 ml of scintillation fluid and kept for 6 h before counting of radioactivity in a scintillation counter. [^{14}C]AP accumulation was expressed as c.p.m./ 10^6 cells per 30 min of incubation.

Assay of peroxidase activity

(a) **Spectrophotometric.** Cells were homogenized in 0.25 M-

sucrose/0.05 M-sodium phosphate buffer, pH 7.2, after freeze-thawing in the presence of cetyltrimethylammonium bromide (final concn. 0.2 %). The peroxidase activity of the cell homogenate or after subcellular fractionation was measured as described previously, by using iodide as an electron donor [7,8].

(b) **^{131}I organification assay.** For ^{131}I organification *in vivo* catalysed by the peroxidase, 10 μ Ci of ^{131}I was injected into each male rat (100–150 g) in the absence or presence of MMI. After a suitable time period, the animal was killed, the stomach contents were washed out with 2 ml of 0.9 % NaCl, and the white cardiac part was discarded. To measure the peroxidase-catalysed organification of ^{131}I , a 10 % (w/v) homogenate was prepared in Tris/sucrose buffer, pH 7.2, centrifuged at 600 g for 10 min, and 1 ml of the supernatant was precipitated with trichloroacetic acid (final concn. 5 %, w/v). The trichloroacetic acid precipitate was washed with 1 ml of cold 5 % trichloroacetic acid containing 1 mM-MMI and 1 mM-KI. Protein-bound ^{131}I in the precipitate was counted in a γ -radiation counter and iodide organification was indicated from the results. The total radioactivity of the precipitate divided by the weight of the tissue (c.p.m./g) denotes the peroxidase-catalysed iodide organification *in vivo*.

For radioiodide organification *in vitro* [6], 2×10^6 cells were added to a 10 ml scintillation vial containing ^{125}I (10^6 c.p.m.) and test drug in a final volume of 1 ml of HBSS with Ca^{2+} (1 mM) and 0.1 % BSA, pH 7.4. Cells were incubated at 37 °C with shaking (120 rev./min) for 30 min, and 0.9 ml samples were then transferred to Microfuge tubes. Cells were pelleted by centrifugation (15000 g -min), lysed with 0.5 ml of distilled water, and TCA was added (final concn. 5 %) to precipitate the protein [BSA (1 mg/ml) was added to help precipitation, if necessary]. After centrifugation (15000 g -min), the trichloroacetic acid precipitate was washed with cold 5 % trichloroacetic acid containing 1 mM-MMI and 1 mM-KI, and the protein-bound iodide (iodide organified by cell peroxidase) was counted for radioactivity in a γ -radiation counter. ^{131}I organified was expressed as c.p.m./ 10^6 cells per 30 min incubation.

Other assays

For estimation of cellular mucin, the cell pellet was suspended in 0.05 M- H_2SO_4 and sonicated at a low energy (two pulses of 10 s duration) in the cold. It was centrifuged (15000 g -min), and the supernatant was used for sialic acid determination by the thiobarbituric acid assay method [20]. For assay of pepsin activity, cells were sonicated in sucrose/phosphate buffer, centrifuged (15000 g -min), and the supernatant was assayed for pepsin activity [21]. Succinate dehydrogenase activity was measured spectrophotometrically [22] in the presence of 4 mM-EDTA, and acid phosphatase activity was measured by the method of Michell *et al.* [23], with β -glycerophosphate as substrate. Protein was determined by the method of Lowry *et al.* [24]. Flavin mono-oxygenase activity was assayed by the method of Poulsen *et al.* [25].

Measurement of HCl secreted

Gastric fluid was collected as described previously [4]. Male rats (100–150 g) fasted for 24 h were injected with MMI or vehicle (water) intraperitoneally. After a suitable time, the animals were killed by cervical dislocation, the abdomen was opened and the gastric fluid was collected by flushing the stomach cavity with 2 ml of 0.9 % NaCl. The stomach washings were centrifuged at 500 g for 10 min in a Sorvall RC5B centrifuge. The clear supernatant was collected, and the amount of fluid was measured to calculate the volume of fluid secreted. For determination of HCl secreted, 1 ml of clear supernatant was titrated with 1 mM-NaOH to pH 6.5 by using an automated burette and pH-stat from Radiometer, Copenhagen, Denmark. The result was

expressed as the total amount of HCl (μmol) secreted in the gastric fluid. ^{131}I may be administered in the presence or absence of MMI to assay organification by gastric peroxidase *in vivo* as described above. For measurement of acid secretion *in vitro*, a chambered gastric mucosal preparation was used as described previously [26], with slight modification. After removal of the outermost serous layer from the fundic stomach gently with the help of fine scissors, the mucosa was kept immersed for 20 min in serosal nutrient solution (HBSS without K_2HPO_4 , pH 7.0) saturated with 100% O_2 . The mucosa was then tied over one end of a plastic tube (10 cm $\times$ 1.5 cm) with the mucosal surface facing out. The area of the mounted mucosa was 1.7 cm². The chambered mucosa was immediately immersed in a small beaker containing 5 ml of unbuffered mucosal solution (HBSS, lacking phosphates and bicarbonate; ionic strength adjusted with NaCl). Drug (MMI) was applied to the serosal solution bubbled with 100% O_2 continuously. The solution was changed every 30 min. The mucosal solution was collected at a specified time (quickly supplementing the system with another 5 ml of fresh mucosal solution), the pH was monitored and the acid output was measured as nmol of H^+ secreted. To measure the corresponding ^{131}I organification by peroxidase, ^{131}I (4×10^6 c.p.m.) was also added to the serosal solution in the presence or absence of MMI. After the desired time, the mucosa was detached, washed with 0.9% NaCl, blotted dry and weighed. The tissue was processed as described above for ^{131}I organification *in vivo*.

Subcellular fractionation

Parietal cells were suspended in 0.25 M-sucrose/0.05 M-Tris/HCl buffer, pH 7.2, and kept in a deep-freeze (-20°C) overnight. After thawing, the cells were hand-homogenized in a glass homogenizer gently. The homogenate was centrifuged at 600 *g* for 10 min, the pellet (cell debris and nuclear fraction) was kept and the supernatant was centrifuged at 12000 *g* for 15 min in a refrigerated Sorvall RC5B centrifuge at 4°C . The resulting pellet was termed the 12000 *g* pellet (crude mitochondrial fraction) and the supernatant the post-12000 *g* supernatant. Peroxidase, succinate dehydrogenase and acid phosphatase activities were assayed in all the fractions.

Since the 12000 *g* pellet is a crude mitochondrial fraction, it was further fractionated in a discontinuous sucrose gradient formed by 1.5 ml each of 34%, 50% and 68% sucrose containing 50 mM-Tris/HCl buffer, pH 7.2. The gradient was centrifuged at 105000 *g* for 90 min in a Beckman ultracentrifuge, in a swing-out rotor at 4°C . The 12000 *g* pellet was resolved into several bands (S_1 , S_2 , S_3 , S_4 and S_5) as shown in Fig. 1(b). Pure mitochondrial fraction was identified by specific marker enzyme assay.

Statistical evaluation

All the data presented are means $\pm$ S.E.M.

RESULTS

Effect of MMI on gastric acid secretion and peroxidase activity of gastric mucosa

MMI administered to rats induced gastric acid secretion dose-dependently, as shown in Fig. 2. Acid secretion was almost linear up to a dose of 2 mg of MMI, above which acid secretion did not increase further significantly. The control rats secreted $24 \pm 4 \mu\text{mol}$ of H^+ in 2.5 h, whereas MMI (2 mg)-treated rats secreted $64 \pm 9 \mu\text{mol}$ of H^+ under identical conditions. The inset of Fig. 2 shows the correlation between time-dependent acid secretion with inhibition of gastric peroxidase activity after administration of 3 mg of MMI. The data indicate that the increase in acid secretion with time has a similar profile to the decrease in peroxidase activity. However, although 50% increase

in acid secretion is associated with 50% decrease in peroxidase activity at 60 min, 60% inhibition of peroxidase activity appears to be sufficient to attain a maximum rate of acid secretion at 120 min. Fig. 3 shows the effect of MMI *in vitro* on the acid secretion by isolated gastric mucosa. The acid secretion increases almost linearly with increasing concentrations of MMI, showing a plateau above 600 μM . The inset further shows the correlation of acid secretion with peroxidase activity against time. The data

Fig. 2. Effect of MMI *in vivo* on gastric acid secretion and peroxidase activity

Various doses of MMI were administered intraperitoneally to 100–150 g male rats fasted for 24 h. Gastric fluid was collected after 2.5 h by washing with 2 ml of 0.9% NaCl. After centrifugation, the clear supernatant was assayed for HCl secreted. The inset shows the correlation of acid secretion and peroxidase activity against time after administration of 3 mg of MMI. The values for acid secretion were plotted after correction for basal secretion. $n = 6-8$.

Fig. 3. Effect of MMI *in vitro* on acid secretion and peroxidase activity of isolated gastric mucosa

Acid secretion was measured in the mucosal solution after addition of various concentrations of MMI as indicated. The values presented are the means of two experiments. The inset represents the correlation between acid secretion and peroxidase activity against time after addition of 450 μM -MMI in the serosal solution. Each value of acid secretion was corrected for basal acid output at the time indicated. $n = 6$.

Fig. 4. Flavin mono-oxygenase activity in stomach (○) and liver (●)

Flavin mono-oxygenase activity was measured with 0.3 mg of liver microsomes or 0.4 mg of stomach microsomes in a final volume of 3 ml containing 0.1 M-phosphate buffer, pH 8.4, 1 mM-NADPH and 1 mM-MMI. NADPH oxidation was measured at 340 nm.

clearly indicate that the time-dependent secretion of acid induced by MMI is associated with a concomitant decrease in peroxidase activity. However, unlike in studies *in vivo*, 75% decrease in peroxidase activity is required to attain the maximum acid secretion. To find whether this acid secretion effect is mediated by the oxidation product of MMI catalysed by the microsomal flavin mono-oxygenase system, this enzyme activity was assayed in the gastric mucosa. Fig. 4 shows that stomach does not contain any appreciable activity, whereas the enzyme is very active in liver. This excludes the possibility of any significant oxidation of MMI in stomach. As gastric mucosa is a multicellular tissue, the precise cellular localization of the peroxidase was therefore studied to seek the initial target of MMI for induction of gastric acid secretion.

Isolation and characterization of peroxidase-enriched cells

During sequential enzymic digestion of the fundic mucosa under controlled conditions, different types of cells were released and isolated. These cells were characterized both microscopically and biochemically. Mucin-secreting cells, chief cells and parietal cells were judged by mucin content, pepsin activity and histamine-stimulated [¹⁴C]AP uptake respectively. These studies indicate that the F₁ fraction was mainly mucin-secreting cells (Table 1). Some chief cells were also present in this fraction, as shown by pepsin activity (Table 1). [¹⁴C]AP uptake in this fraction was not studied, as the cells did not appear morphologically to be parietal cells. The F₂ fraction was a mixture of the above-mentioned cells containing nearly 30% parietal cells as observed microscopically

and showing 44% stimulation of [¹⁴C]AP uptake by histamine. This fraction, however, contains very high peroxidase activity. Upon density-gradient centrifugation in Percoll, the mixed cell population of the F₂ fraction was resolved into four cell fractions, designated B₁, B₂, B₃ and B₄ (Fig. 1a). Cells of fraction B₁ appeared to be parietal cells, as evidenced by histamine-stimulated [¹⁴C]AP uptake (Table 1). The cells also lacked pepsin activity and mucin, indicating the absence of chief and surface epithelial cells. Some unidentified small cells (endocrine cells) may be present in this fraction. However, the total number of parietal cells enriched in this fraction was low, and parietal cells of smaller diameters (< 12 μm) were found to be concentrated in this fraction. About 60% of the loaded cells on the Percoll gradient were concentrated in the B₂ fraction. Cells of this fraction were readily identified as parietal cells, as judged by published morphological criteria [27–29], by their comparatively larger diameter (> 12 μm), concentric nuclei and eosinophilic cytoplasm. These cells exhibited histamine-stimulated [¹⁴C]AP uptake and increased O₂ consumption in the presence of carbachol, isobutylmethylxanthine and histamine (Fig. 5). A few pepsin- and mucin-secreting cells were observed in this fraction. The yield of the purified parietal cells in fractions B₁ and B₂ together was calculated as (2.5–3) × 10⁶ cells per rat stomach. Cells in combined B₁ and B₂ fraction were 70% pure parietal cells, and about 90% of the cells excluded Trypan Blue. Cells in fractions B₃ and B₄ were very small in diameter (< 8 μm) compared with those in fractions B₁ and B₂. These were mainly pepsinogen-secreting chief cells. However, fraction B₄ was contaminated with mucin-secreting cells and broken parietal cells. [¹⁴C]AP uptake was not measured in this fraction, as it contained mostly broken parietal cells. When peroxidase activity was assayed biochemically [7,8], about 25% of peroxidase activity in combined fractions F₁ and F₂ was recovered in fraction F₁ (Table 1), whereas about 90% of the activity of the F₂ fraction was present in the combined B₁ and B₂ fraction. However, only the B₂ fraction contains nearly 66% of the activity of the F₂ fraction. No significant activity was found in fractions B₃ and B₄. These results indicate that peroxidase is present mainly in the parietal cells which could be recovered in fractions B₁ and B₂. The peroxidase activity in fraction F₁ may be due either to contamination of some B₁ and B₂ cells in this fraction or to some endogenous peroxidase activity of the surface epithelial cells. When parietal cells (fractions B₁ + B₂) were treated with a histochemical probe for peroxidase activity such as diaminobenzidine and H₂O₂, an intense black deposition of oxidized diaminobenzidine was observed inside the cells. No such deposition was observed in cells with diaminobenzidine without H₂O₂. This histochemical study supports the location of

Table 1. Cellular localization of gastric peroxidase

Values are means ± S.E.M. for 3–6 experiments.

Cell fraction	Peroxidase		Mucin		Pepsin		10 ⁻³ × [¹⁴ C]AP uptake/10 ⁶ cells (c.p.m.)	
	Total activity (unit)	Specific activity (units/mg)*	Total (μg)	(μg/mg)*	Total activity (unit)	Specific activity (units/mg)*	– Histamine	+ Histamine
F ₁	65 ± 3	18 ± 4.3	21 ± 1	5.81 ± 1.3	18 ± 4	4.98 ± 1.7		
F ₂	191 ± 5	40 ± 6.8	5 ± 1	1.04 ± 0.22	53 ± 8	11 ± 2	3.4 ± 0.2	4.9 ± 0.5
B ₁	44 ± 16	71 ± 14	0.5 ± 0.06	0.8 ± 0.2	0	0	5.9 ± 0.7	12.4 ± 2.5
B ₂	125 ± 5	80 ± 7	0.9 ± 0.2	0.58 ± 0.2	7 ± 2	4.48 ± 1.06	6.3 ± 0.8	12.8 ± 1.4
B ₃	3 ± 0.5	5 ± 1.5	0.6 ± 0.04	1 ± 0.3	9 ± 1	15 ± 2.5	4.6 ± 0.4	4.6 ± 0.2
B ₄	14 ± 1	17 ± 2.5	2.4 ± 0.5	2.93 ± 1	3 ± 1	37.80 ± 3.8		

* mg of cell protein.

Fig. 5. Effect of carbachol, isobutylmethylxanthine (IBMX) and histamine on O₂ consumption by isolated cells of fraction B₂.

At 30 min after cell isolation, 10⁶ cells were added in a final volume of 2 ml of HBSS with BSA (0.1%) and Ca²⁺ (1 mM) to the flask of the Gilson Oxygraph, and O₂ consumption was measured in the absence and presence (0.1 mM) of the test drug. *n* = 3–5.

peroxidase activity in the interior of the parietal cells. For all biochemical studies the combined B₁ and B₂ fractions were used.

Subcellular localization of peroxidase in the parietal cells

In order to localize the peroxidase activity in the parietal cells, subcellular fractionation of the parietal-cell homogenate was

carried out. About 70% of the activity of the parietal-cell homogenate was enriched in the 12000 *g* pellet, constituting the crude mitochondrial fraction (Table 2), which was positive for succinate dehydrogenase activity (marker for mitochondria). Some acid phosphatase activity (marker for lysosomes) is also present in the fraction, owing to the presence of some unbroken lysosomes. However, the post-12000 *g* supernatant was enriched with the lysosomal acid phosphatase activity, which may be due to breakage of the fragile lysosomal membrane during homogenization. For further precise localization of the peroxidase, the 12000 *g* pellet was fractionated on a discontinuous sucrose gradient (Fig. 1b). The 12000 *g* pellet was resolved into five bands, designated S₁, S₂, S₃, S₄ and S₅. Of these, S₄ appeared to constitute the major part of the organelles, and about 76% of the peroxidase activity of the 12000 *g* pellet was enriched in this fraction (Table 3). The S₄ fraction was also enriched in succinate dehydrogenase activity. This indicates that purified mitochondria were concentrated in this S₄ fraction appearing at the 50/68%-sucrose interface. Some lysosomes present in the 12000 *g* pellet are distributed in fractions S₂–S₅, with highest specific activity in the S₃ fraction. The identity of the small amount of organelle in S₁ and S₅ fractions is not known. A small amount of peroxidase activity in the S₅ fraction may be due to contamination by some mitochondria from the S₄ fraction. It thus appears that parietal-cell mitochondria are the site of the peroxidase activity. As parietal cells are characterized by the presence of a large number of mitochondria compared with other cells, owing to energy-requiring active transport of H⁺, the probability may exist that enriched peroxidase activity in these cells may be due to enrichment with mitochondria. The data in Table 4, however, indicate that the specific activity of the peroxidase is highest only in the parietal cell (fractions B₁ + B₂), whereas the specific activity of succinate dehydrogenase, the marker for mitochondria, is almost constant in all the cell fractions. The very high ratio of peroxidase activity to succinate dehydrogenase activity in the parietal-cell fraction further suggests that the enzyme is specifically induced in the parietal cell.

Table 2. Subcellular localization of peroxidase activity of the parietal cells

Fraction	Peroxidase		Succinate dehydrogenase		Acid phosphatase	
	Total activity (units)	Specific activity (units/mg)	Total activity (unit)	Specific activity (unit/mg)	Total activity (units)	Specific activity (units/mg)
Parietal-cell homogenate	150	69.4	0.68	0.3	4.2	2
600 <i>g</i> pellet	10	10.4	0	0	0.4	0.4
12000 <i>g</i> pellet	105	131.3	0.64	0.8	0.8	1.0
Post-12000 <i>g</i> supernatant	0	0	0	0	2.0	8.7

Table 3. Purification of peroxidase-enriched organelles from 12000 *g* pellet on discontinuous sucrose gradient

Fraction	Peroxidase		Succinate dehydrogenase		Acid phosphatase	
	Total activity (units)	Specific activity (units/mg)	Total activity (unit)	Specific activity (unit/mg)	Total activity (units)	Specific activity (units/mg)
12000 <i>g</i> pellet	105	131.3	0.64	0.8	0.8	1.0
S ₁	0	0	0	0	0	0
S ₂	0	0	0.02	0.5	0.1	1.4
S ₃	0	0	0.01	0.2	0.2	3.8
S ₄	79.5	176.7	0.53	1.2	0.3	0.7
S ₅	4.1	42	0.05	0.5	0.1	1

Table 4. Specific activity of peroxidase and succinate dehydrogenase in the mitochondrial fraction of isolated cells

Cell fractions	Specific activity		Total peroxidase activity
	Peroxidase (units/mg)	Succinate dehydrogenase (unit/mg)	
F ₁	37.5	0.69	54.35
B ₁ + B ₂	131.3	0.80	164.13
B ₃	15	0.73	20
B ₄	28	0.72	38.88

Fig. 6. Effect of PCMBS and DAS on peroxidase activity of isolated parietal-cell mitochondria

Purified mitochondria (20 μ l, containing 20 μ g of protein) were incubated in the presence of various concentrations of PCMBS ($\blacktriangle$) and DAS ($\bullet$) for 15 min at room temperature before assay of peroxidase activity.

Orientation of peroxidase on the mitochondrial membrane

To investigate the orientation of the peroxidase in the mitochondrial membrane, the purified mitochondria (S₄ fraction) were treated with some membrane-impermeant reagents such as PCMBS and diazonium salt of sulphanilic acid (DAS), prepared just before use in accordance with the published procedure [30]. A dose-dependent inactivation of peroxidase activity was observed with both PCMBS and DAS (Fig. 6). PCMBS was more potent than DAS. At 1 mM, PCMBS could block peroxidase activity completely, whereas the same concentration of DAS could inhibit by only 60%. However, 4 mM-DAS caused almost complete inactivation of the enzyme. Inactivation of the peroxidase activity by these membrane-impermeant reagents indicates that the peroxidase is localized on the outer membrane of the mitochondria, with its catalytic site oriented outside.

Effect of MMI on the peroxidase activity of isolated parietal cells

To determine whether MMI-induced acid secretion is associated with the inhibition of the peroxidase activity of the parietal cells, the ¹³¹I organification in these cells was studied in the presence and absence of MMI. The peroxidase activity in the parietal cell was found to be inhibited in a concentration-dependent fashion when incubated with various concentrations of MMI (Fig. 7a). MMI at 0.1 mM almost completely inhibited the peroxidase-catalysed organification. The enzyme activity was inhibited by 50% at 0.1 μ M-MMI, indicating that MMI is a potent inhibitor of the parietal-cell peroxidase. Peroxidase activity of the mitochondrial fraction of the parietal cells was also strongly inhibited *in vitro* in the presence of MMI when the

Fig. 7. Effect of various concentrations of MMI on peroxidase activity (a) of isolated parietal cells as measured by ¹³¹I organification, and (b) of parietal-cell mitochondrial fraction

Detailed procedures are described in the Materials and methods section.

activity was assayed with iodide as an electron donor (Fig. 7b). MMI at 0.1 mM concentration completely inhibited the peroxidase activity. Unlike in cells, MMI at 0.1 μ M did not affect the enzyme activity of the mitochondrial fraction. This may be due to the presence of a higher concentration of iodide in the assay system, which may protect the enzyme against inhibition caused by MMI binding at relatively lower concentration. MMI also inhibits the peroxidase activity of the F₁-cell mitochondria. Whether this effect is shown by the contaminating parietal cell in this fraction or by the endogenous peroxidase activity of the surface epithelial cells, if any, cannot be answered until pure surface epithelial cells are obtained.

DISCUSSION

We have presented evidence showing that MMI induces gastric acid secretion in experiments both *in vivo* and *in vitro*, and the increase in acid secretion correlates well with the decrease in the peroxidase activity. That MMI is directly stimulating acid secretion without being metabolized to some active compound *in vivo* is evident from its direct effect *in vitro* on isolated gastric mucosa. MMI is known to be oxidized by the flavin mono-oxygenase system, and MMI oxidation is widely used as a marker for this enzyme activity [25,31,32]. Our studies indicate that gastric mucosa contains no significant amount of flavin mono-oxygenase activity, but liver does. Thus the possibility of the induction of acid secretion by any oxidation product of MMI formed by flavin mono-oxygenase activity of the stomach may be excluded. It is thus reasonable to suggest that the effect is mediated directly by MMI through inhibition of gastric peroxidase activity. As acid secretion is a specific function of the

parietal cell, it is highly probable that peroxidase should be located in these cells if it has any role in the control of acid secretion. Our results indicate that peroxidase is an endogenous protein of the parietal cell mainly, although very small activity may be present in the surface epithelial and chief cells. Some contamination by a few parietal cells in these cell fractions is also evident in microscopic examination. Both B_1 ($< 12 \mu\text{m}$) and B_2 ($> 12 \mu\text{m}$) fractions of parietal cells contain this enzyme, as revealed by biochemical and histochemical techniques. Small parietal cells may belong to the deeper level of the gastric mucosa, whereas bigger cells may come from the superficial level [33]. The highest activity of the peroxidase in $B_1 + B_2$ fractions is not due to higher cell number. The activity per mg of cell protein is highest in this combined parietal-cell fraction, suggesting that it is specifically located in these cells. As the enzyme is localized in the mitochondria, which are numerous in the parietal cell to cope with the demand of energy for active transport of H^+ , the high peroxidase activity may be due to the larger amount of mitochondria in these cells. However, the activity when calculated per mg of mitochondrial protein of isolated cell fraction (specific activity) was found to be highest in the parietal-cell fraction, indicating its enrichment in these cells only. The higher ratio of total peroxidase activity to total succinate dehydrogenase activity further indicates that peroxidase is specifically induced in the mitochondria of the parietal cell. The outward orientation of the catalytic site of this enzyme in the outer membrane of mitochondria is evident from the inactivation of the enzyme by the membrane-impermeant reagents PCMBs and DAS. These highly charged molecules (hence membrane-impermeant) are known to bind covalently with the thiol group and the tyrosine moiety of the protein [30,34], and pure gastric peroxidase is also known to contain some active thiol groups [7]. It appears that the cytosolic orientation of its catalytic site may act as an effective system for its quick interaction with H_2O_2 to perform some specialized function.

The physiological function of the gastric peroxidase is not yet known. The antibacterial activity of the peroxidases of saliva and milk [35] and bactericidal activity of neutrophil and eosinophil peroxidases [36,37] have been documented. The ovoperoxidase of sea-urchin eggs helps in the formation of the 'fertilization membrane' to prevent 'polyspermy' during fertilization [38,39]. The intestinal peroxidase and oestrogen-induced uterine peroxidase are contributed by the invading eosinophil [40,41], and may exert bactericidal and antiparasitic activity in intestine [37] or in the catabolism of oestradiol in the uterus [41]. As gastric peroxidase is located in the parietal cell, its possible involvement in the acid secretory function may be expected. Activation of the parietal cell with various secretagogues to induce acid secretion is accompanied by increased O_2 consumption [42] with similarity to that of the 'respiratory burst' [43] of the phagocytes or ovum-sperm interaction [38,39]. 'Respiratory burst' is associated with increased substrate metabolism and generation of H_2O_2 [38,44,45]. Peroxidases in the phagocytic cells utilize H_2O_2 to oxidize Cl^- and SCN^- to form hypochlorous acid or hypothiocyanite ion, which have very strong bactericidal-antimicrobial activity [36,37]. Similarly, ovoperoxidase utilizes H_2O_2 for cross-linking the tyrosine residues of the membrane protein to cause hardening of the 'fertilization membrane' [38,39]. Phagocytosis and fertilization are also associated with increased hexose monophosphate shunt activity [43] and acid production [46,47]. During active transport of H^+ by activated parietal cells, increased O_2 consumption may lead to generation of intracellular H_2O_2 . As a very high concentration of H_2O_2 is toxic to the cell, parietal-cell peroxidase may utilize this oxygen metabolite to perform some specialized function. We suggest that parietal-cell peroxidase plays an important role in maintaining the normal

acid-secretory function of the activated parietal cells by eliminating the increased intracellular H_2O_2 , which may otherwise damage the machinery for acid secretion. As the MMI effect is mediated through the inhibition of gastric peroxidase, it is tempting to suggest that accumulated intracellular H_2O_2 , before reaching the 'critical' damaging level, either may act like the second-messenger system of the parietal cell or may stimulate its stimulus-secretion coupling system to induce acid secretion. Various possibilities of MMI-induced acid secretion have been proposed [48]. The effect of MMI directly on the isolated parietal cell should be investigated, with emphasis on parietal-cell activation-associated 'respiratory burst', intracellular H_2O_2 generation and its role in the induction of acid secretion.

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