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STUDIES ON THE MECHANISM OF ACTION OF THE OMEPRAZOLE-DERIVED CYCLIC SULPHENAMIDE

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Abstract—The inhibitory effects of omeprazole and omeprazole-derived metabolites were studied on *Escherichia coli* glutaminase activity at pH 2.5 which might represent the conditions present at the target enzyme (K^+/H^+–ATPase) in the secretory membrane of the intact parietal cell. Omeprazole and the omeprazole-derived cyclic sulphenamide inhibited glutaminase at pH 2.5 with identical potency (IC_{50} 36 μM). The substrate, glutamine as well as the mercaptane, dithiothreitol, protect the enzyme. Furthermore, dithioerythritol was found to reverse inhibition. This indicates that an SH-group localized in the substrate binding center of glutaminase is most likely involved in the reaction leading to enzyme inhibition. Glutaminase inhibition by both compounds was less pronounced at pH 5.0. Omeprazole radical, the metabolite generated from the cyclic sulphenamide at more neutral pH values, failed to affect the enzyme. These findings were in contrast with the properties of the omeprazole-derived cyclic sulphenamide and radical at the K^+/H^+–ATPase preparation. This enzyme was inhibited by both compounds at pH 7.5 with a high potency, and reversal experiments with dithiothreitol demonstrate that these agents interfere with SH-groups of the K^+/H^+–ATPase. From these data it is suggested that the cyclic sulphenamide and the radical interfere by different reaction pathways with enzymatic SH-groups.

The substituted benzimidazole, omeprazole, inhibits gastric acid secretion by suppressing K^+/H^+–ATPase [1], the enzyme which represents the terminal step of H^+ production by the parietal cell [2]. Several studies with purified K^+/H^+–ATPase preparations have shown that the inhibitory potency of omeprazole is enhanced in an acidic environment [1, 3], and it was found that the H^+-activated derivatives of omeprazole have the ability to react with SH-groups of the K^+/H^+–ATPase [4]. Different proposals have been made for the chemical structure of the acid-induced transformation products of substituted benzimidazoles as well as for the nature of their reactions with SH-groups of the K^+/H^+–ATPase [5, 6] (see Fig. 1). Recently, Figala *et al.* [6] have shown that omeprazole rearranges at pH 1.0 in a first step to a cyclic sulphenamide (compound II, Fig. 1). This cyclic sulphenamide decomposes with increasing pH values to a tetracyclic radical (compound III, Fig. 1), a decomposition product which inhibits the K^+/H^+–ATPase with high potency [3].

K^+/H^+–ATPase activity has to be assayed at neutral pH-values and therefore cannot be used for experiments with the cyclic sulphenamide. *Escherichia coli* glutaminase is an enzyme which has a broad pH optimum between 3.5 and 5.0 and is susceptible to inhibition by the SH-reagent 2-mercuribenzoate [7]. For this reason we have used this enzyme for studying the effects of omeprazole, the omeprazole cyclic sulphenamide and radical on enzyme activity at low pH conditions.

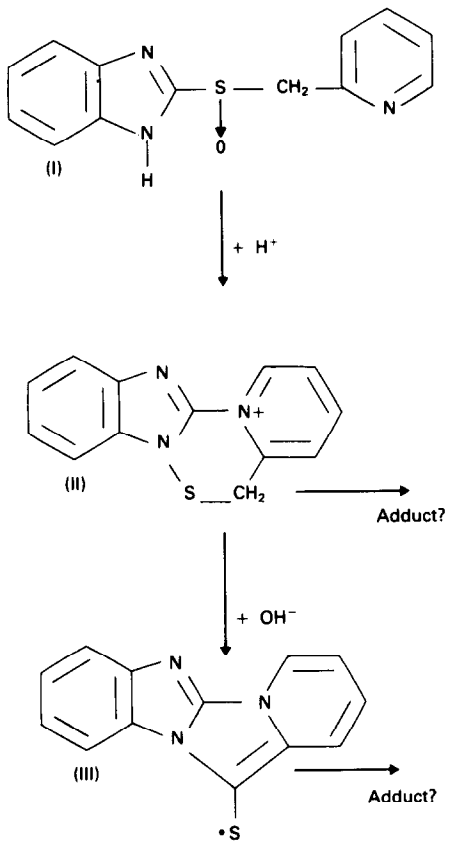

Fig. 1. Reaction pathways of omeprazole according to Figala *et al.* [6].

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MATERIALS AND METHODS

Experiments with glutaminase (EC 3.5.1.2.). Glutaminase was assayed in 0.5 ml samples containing 100 mM citrate buffer pH 2.5 or 5.0, 20–40 μg protein and 40 mM glutamine for 15 min at 22°. The reaction was stopped by adding 0.5 ml 20% ice-cold trichloroacetic acid. After centrifugation, 0.5 ml samples were mixed with 5 ml reagent grade water and liberated ammonia was measured with Nessler's reagent. Control reaction rates were 6.2 ± 0.3 U/mg protein and 8.6 ± 0.09 U/mg protein at pH 2.5 and 5.0 respectively (mean ± SEM from three determinations). At pH 7.0 the enzyme is inactive. For inhibition experiments the following stock solutions were prepared: (a) omeprazole in methanol pH 7.0; (b) omeprazole radical in dimethylsulphoxide pH 7.0; (c) omeprazole cyclic sulphenamide in methanol pH 1.0. Glutaminase (20 μg protein) was incubated with indicated inhibitor concentrations in 0.25 ml citrate buffer pH 2.5 or 5.0 at 22° for 15 min. The reaction was initiated by adding 0.25 ml 80 mM glutamine dissolved in citrate buffer pH 2.5 or 5.0. When glutaminase was preincubated at pH 7.0 with the inhibitors tested, the remaining enzyme activity was determined after back titration of the samples to pH 5.0 with the substrate solution.

Reversal experiments with dithioerythritol (DTE). Glutaminase (200 μg protein) was incubated with 50 μM of the omeprazole cyclic sulphenamide in 10 mM citrate buffer pH 2.5. After 15 min the pH was back titrated with 200 mM Tris–HCl buffer pH 7.5 to pH 7.0. Then DTE (final concentration 25 mM) was added and was allowed to react for 2 hr. The samples were then dialysed for 4 hr against 100 mM citrate buffer pH 7.0 to remove DTE. The remaining enzyme activity was determined at pH 5.0.

Determination of SH-groups. One mg glutaminase was incubated in 1 ml citrate buffer pH 7.0 with 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), in the absence or presence of 1% sodium dodecyl sulphate (SDS). After 30 min the protein was removed by pressing the samples through a Millipore filter (pore size: 0.22 μm) in a Swinnex filter system. Absorbance of 5-thio-2-nitrobenzoic acid was measured at 412 nm and the amount of SH-groups was calculated using a molar absorption coefficient of 13600.

Experiments with K^+/H^+*–ATPase*. K^+/H^+–ATPase was purified by differential and density gradient centrifugation of a guinea-pig mucosal cell homogenate as described previously [8].

Measurement of K^+/H^+*–ATPase activity*. K^+/H^+-ATPase was assayed in 1 ml samples containing 2 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5), 10 μg membrane protein in the absence or presence of 5 mM KCl. The reaction was initiated by addition of ATP (final concentration 2 mM) and was stopped after 15 min incubation at 37° with 1 ml 1 N HCl. Liberated P_i was measured in 0.5 ml aliquots according to the method of Carter and Karl [9]. Control reaction rates were 14.8 ± 1.0 μmol P_i/mg protein × hr in the absence and 57.5 ± 5.0 μmol P_i/mg protein × hr in the presence of KCl (mean ± SEM of three different enzyme preparations). For inhibition experiments the following stock solutions were prepared: (a) omeprazole cyclic sulphenamide in methanol pH 1.0; (b) omeprazole radical in dimethylsulphoxide pH 7.0; (c) the mother compound omeprazole was degraded in methanol pH 1.0 for 15 min and was then added to the enzyme buffered at pH 7.5. All inhibitors were allowed to react for 30 min at 22°, with the enzyme buffered at 7.5, and the remaining enzyme activity was then determined as described above.

Determination of SH-groups. SH-groups at the K^+/H^+–ATPase were determined as described for the glutaminase with the modification that 0.5 mg membrane protein was incubated in 1 ml 50 mM Tris–HCl buffer pH 7.5 containing 2 mM $MgCl_2$ and 5 mM KCl with or without 1% SDS.

Chemicals and drugs. Collagenase (125–230 units/mg protein), Na_2–ATP, glutaminase, dithiothreitol, dithioerythritol, *p*-hydroxymercuribenzoate, *o*-iodosobenzoic acid, *N*-ethylmaleimide, 5,5′-dithiobis (2-nitrobenzoic acid) (Sigma, Munich); pronase E (70,000 PUK/g protein), Nessler's reagent (Merck Darmstadt); omeprazole was kindly donated by Dr. E. Carlsson, AB Hässle Mölndal, Sweden; the omeprazole cyclic sulphenamide was kindly donated by Prof. Klemm, Byk Gulden Konstanz, F.R.G.; the omeprazole radical was kindly donated by Dr. G. Rackur, Hoechst AG, F.R.G.

RESULTS

Inhibition of glutaminase by omeprazole, the omeprazole-derived cyclic sulphenamide and radical

Inhibition of glutaminase by omeprazole and the omeprazole cyclic sulphenamide was both pH- and concentration-dependent. After 15 min preincubation at pH 2.5, both compounds inhibited the enzyme with an IC_{50} of 36 μM (Fig. 2a, b). The presence of 40 mM glutamine totally prevented inactivation of the enzyme. At pH 5.0 both omeprazole and the cyclic sulphenamide were less potent (Fig. 2a, b) and at pH 7.0 both compounds were inactive.

The omeprazole-derived tetracyclic radical failed to affect glutaminase activity at all three pH values tested (data not shown).

Preincubation of glutaminase with equimolar concentrations of omeprazole and the omeprazole-derived cyclic sulphenamide at pH 2.5 shows congruent time-dependent inactivation of the enzyme by both compounds (Fig. 3).

Protection with dithiothreitol (DTT)

When glutaminase was incubated with 1 mM DTT alone the enzyme was slightly activated (+11%). Higher DTT concentrations could not be tested since they interfere with the ammonia assay. At pH 2.5 DTT protected glutaminase in a concentration-dependent manner from inhibition by 100 μM omeprazole or the omeprazole-derived cyclic sulphenamide (Fig. 4).

Reversal studies with dithioerythritol (DTE)

To investigate whether the mercaptane DTE was able to restore the already inhibited enzyme, glutaminase was incubated at pH 2.5 for 15 min with 50 μM of the omeprazole-derived cyclic sulphen-

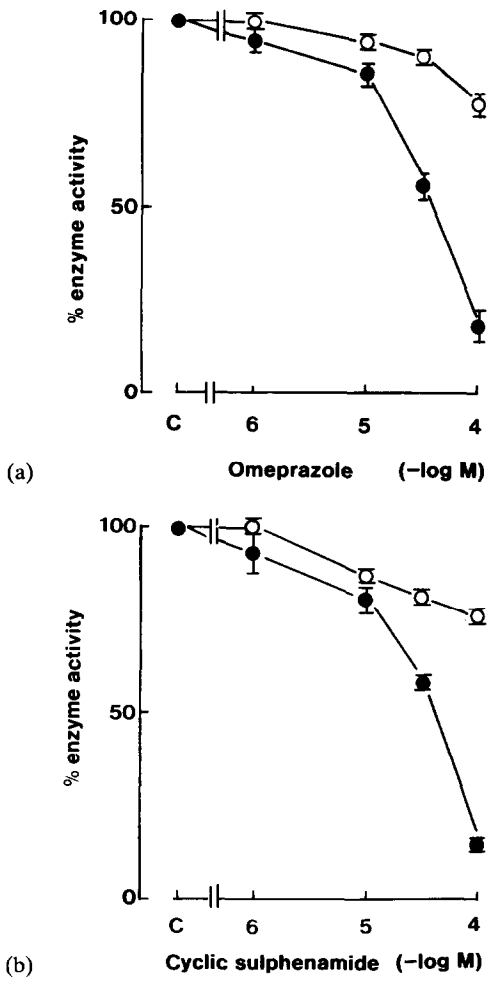

Fig. 2. Concentration–response curves for inhibition of *Escherichia coli* glutaminase by omeprazole (a) and omeprazole cyclic sulphenamide (b). Glutaminase (20 μg protein) was incubated with indicated inhibitor concentrations for 15 min at pH 2.5 (●) or 5.0 (○). Remaining enzyme activity was then determined as described in Methods. The enzyme activity in the absence of the inhibitor was set at 100%. Values are mean ± SEM from three determinations.

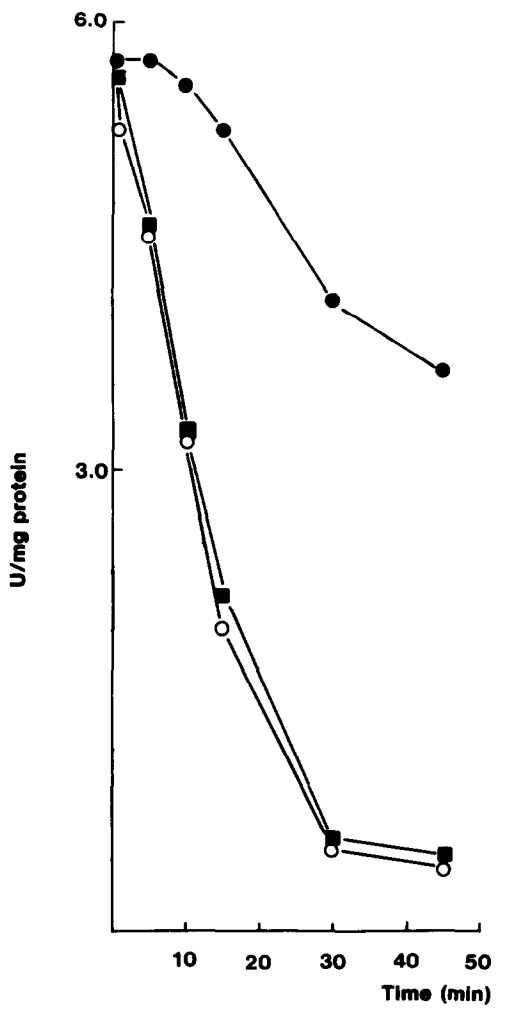

Fig. 3. Time–course for inactivation of glutaminase by 50 μM omeprazole and omeprazole cyclic sulphenamide. Glutaminase (80 μg protein/ml) was incubated at pH 2.5 in the absence (●) or presence of 50 μM omeprazole (○) and omeprazole cyclic sulphenamide (■). Each time point is the mean of three different determinations. SEM were less than 10%.

Table 1. Reactivation of inhibited glutaminase by dithioerythritol (DTE)

Conditions		Enzyme activity (U/mg protein)
A	Enzyme 15 min at pH 2.5, then pH 7.0 25 mM DTE for 2 hr, dialysis	9.3 ± 0.27
B	Enzyme + 50 μM cyclic sulphenamide 15 min at pH 2.5, then pH 7.0, 25 mM DTE for 2 hr, dialysis	7.5 ± 0.15
C	Enzyme + 50 μM cyclic sulphenamide 15 min at pH 2.5, then pH 7.0, dialysis	4.2 ± 0.09

Glutaminase (200 μg protein/ml) was incubated in the absence (A) or in the presence of 50 μM of the omeprazole cyclic sulphenamide (B, C) in 10 mM citrate buffer pH 2.5. After 15 min in all three samples the pH was back-titrated to pH 7.0 and DTE (final concentration 25 mM) was added (A, B). Thereafter, the samples were dialysed for 4 hr and the remaining enzyme activity was determined at pH 5.0 (values: mean ± SEM from three experiments).

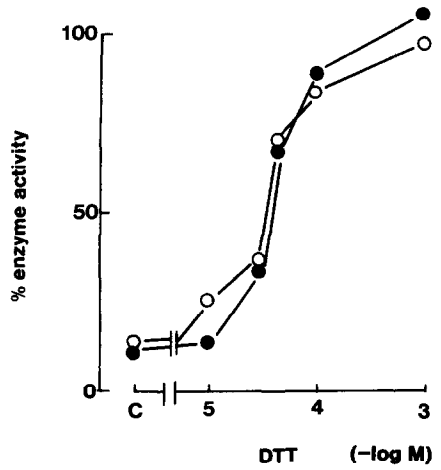

Fig. 4. Effect of dithiothreitol (DTT) on the inhibitory action of omeprazole and omeprazole cyclic sulphenamide. Glutaminase (20 μg protein) was incubated with increasing concentrations of DTT at pH 2.5 with 100 μM omeprazole (●) and omeprazole cyclic sulphenamide (○) for 15 min. Subsequently, glutaminase activity was determined as described in Methods. Each point is the mean of three determinations. SEM were less than 10%.

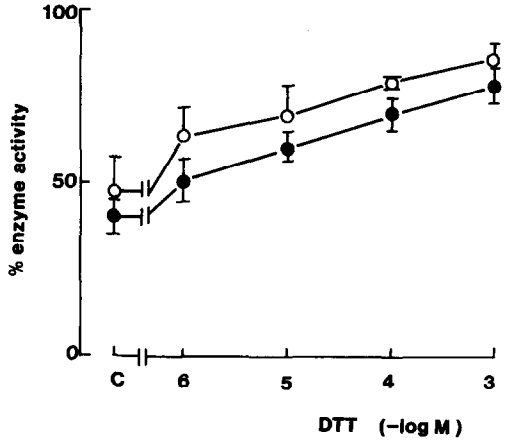

Fig. 5. Reversal of K^+/H^+–ATPase inhibition by DTT. K^+/H^+–ATPase was incubated for 10 min with 1 μM omeprazole cyclic sulphenamide (●) or 0.5 μM omeprazole radical (○). Following this, the concentrations of DTT shown were added and were allowed to react for 20 min. The remaining enzyme activity was determined as described in Methods. Values are mean ± SEM of three different enzyme preparations.

amide which reduced enzyme activity to about 47% of control activity (4.1 ± 0.1 U/mg protein vs 8.6 ± 0.4 U/mg protein). Treatment of the inhibited enzyme with 25 mM DTE for 2 hr as described in Materials and Methods restored the enzyme activity to about 81% of the control activity (Table 1) demonstrating that the mercaptane could reverse the inhibitory action.

Inhibition of K^+/H^+–ATPase by acid-degraded omeprazole, the omeprazole-derived cyclic sulphenamide and radical

The omeprazole-derived cyclic sulphenamide (dissolved in methanol pH 1.0) inhibited the purified K^+/H^+–ATPase after 30 min preincubation at pH 7.5 with an IC_{50} of 1.2 ± 0.37 μM (mean ± SEM from three different enzyme preparations). Acid-degraded omeprazole (pH 1.0 solution of the mother compound) and the omeprazole radical inhibited the enzyme with IC_{50} values of 1.4 ± 0.34 and 0.3 ± 0.03 μM respectively which is in agreement with the data published previously [3].

Reversal experiments with DTT

In order to study whether DTT reverses the inhibitory action of the three compounds on K^+/H^+–ATPase, the enzyme was incubated for 10 min with 1 μM omeprazole or the omeprazole-derived cyclic sulphenamide and 0.5 μM of the omeprazole radical which reduced K^+/H^+–ATPase activity by about 50%. DTT added afterwards and being allowed to react for 20 min partially reversed the inhibitory effect of all compounds in a concentration-dependent manner (Fig. 5) (omeprazole data not shown). It should be noted that DTT alone up to a concentration of 1 mM did not change the K^+-stimulated ATPase activity.

Inhibition of glutaminase and K^+/H^+–ATPase by different SH reagents

The inhibitory effects of increasing concentrations of *p*-hydroxymercuribenzoate, *o*-iodosobenzoic acid and *N*-ethylmaleimide on glutaminase (20 μg protein) and K^+/H^+–ATPase (10 μg protein) preincubated at pH 7.5 for 30 min are summarized in Table 2.

Table 2. IC_{50} values (μM) of *p*-hydroxymercuribenzoate, *o*-iodosobenzoic acid, and *N*-ethylmaleimide to inhibit *Escherichia coli* glutaminase and guinea-pig parietal cell K^+/H^+–ATPase

Reagent	K^+/H^+–ATPase	Glutaminase
p-Hydroxymercuribenzoate	0.6 ± 0.03	5.2 ± 0.25
o-Iodosobenzoic acid	220 ± 46	17,500 ± 500
N-Ethylmaleimide	700 ± 150	>20,000*

* 28% inhibition in the presence of 20 mM NEM.

K^+/H^+–ATPase (10 μg protein) and glutaminase (20 μg protein) were preincubated for 30 min at 22° with at least five different concentrations of each reagent tested. Remaining enzyme activity was determined as described in Methods. Values are mean ± SEM of three different enzyme preparations (K^+/H^+–ATPase) or three different determinations (glutaminase).

Determination of reactive SH-groups of glutaminase and K^+/H^+–ATPase

The amount of free reactive thiol groups at the K^+/H^+–ATPase was 45 ± 0.6 μmol SH/g protein; the total number of SH groups measured in the presence of 1% SDS was 53 ± 0.6 μmol SH/g protein. With glutaminase 11 ± 0.58 μmol SH/g protein was found. In the presence of 40 mM glutamine 1.0 ± 0.25 μmol SH-groups/g protein react. The total number measured in the presence of 1% SDS was 38 μmol SH/g protein.

DISCUSSION

Different methods have been used to study the nature of the interaction of the substituted benzimidazole omeprazole with the target enzyme, K^+/H^+–ATPase under conditions of acidic pH: (a) incubation of the compound at slightly low pH (pH 6.1) together with the enzyme [1]; (b) solution of the compound in strong acid (pH 1.0 or 2.0) before adding the reaction product(s) to the K^+/H^+–ATPase buffered at pH 7.5 [3, 10, 11]; (c) incubation of the compound with intact pumping vesicles resulting in activation of the compound in the acidic vesicle interior [12, 13].

In this study we have used *Escherichia coli* glutaminase, an enzyme which can be assayed at strong acidic conditions for investigating the effect of omeprazole and omeprazole breakdown products on enzyme activity. It was found that omeprazole and the omeprazole-derived cyclic sulphenamide inhibited *Escherichia coli* glutaminase preincubated and assayed at pH 2.5 with identical potency. The time-course of enzyme inhibition followed pseudo-first order kinetics and was identical for both compounds. These data demonstrate that omeprazole and the cyclic sulphenamide have the ability to react with glutaminase and suggest that at pH 2.5 the cyclic sulphenamide is generated from the mother compound. The studies with DTT demonstrate that concentrations of the mercaptone equimolar with those of the inhibitors protect the enzyme from inhibition. Furthermore, high DTE concentrations were able to reactivate the inhibited enzyme, indicating that the omeprazole-derived cyclic sulphenamide binds to SH-groups of the enzyme. Nevertheless, the character of glutaminase as a thiol enzyme is not at all certain [14]. Hartmann [14] has found, in agreement with our data, that low concentrations of organic mercurials are able to inhibit glutaminase, whereas iodoacetate (5 mM) and *N*-ethylmaleimide (4 mM) were without effect. We found that *o*-iodosobenzoic acid and *N*-ethylmaleimide inhibited the enzyme, but only in concentrations which might not be specific for SH-groups. However, 11 μmol free reactive thiol groups per gram glutaminase were quantified with the DTNB method. On the basis of a molecular weight of 110,000 [14] approximately 1 mol free SH per mol enzyme can be calculated. The substrate, glutamine, was found to protect the enzyme as well as the mercaptane, DTT, and the number of reacting thiol groups was reduced by 40 mM glutamine. These data suggest that omeprazole and the omeprazole-derived cyclic sulphenamide inactivate glutaminase by forming a disulphide bond between an SH-group located in the substrate-binding center of the enzyme and the compound.

With increasing pH values in the preincubation medium (pH 5.0 and 7.0) the cyclic sulphenamide gradually lost its inhibitory potency on glutaminase. At these more neutral pH values the cyclic sulphenamide decomposes to the tetracyclic radical [6] (see Fig. 1). The omeprazole radical itself failed to inhibit glutaminase. These findings contrast totally with the effects of the cyclic sulphenamide and the radical on the K^+/H^+–ATPase, assayed at pH 7.5. The interaction studies with DTT demonstrate that the mercaptane can reverse the inhibitory action of the omeprazole radical at the K^+/H^+–ATPase. Thiyl radicals have the ability to interfere with enzymatic SH-groups by different reactions as shown in the following scheme:

$$\text{I}\quad R\text{-SH} + X\text{-}S\cdot \rightarrow R\text{-}S\text{-}S\text{-}X\cdot$$

or

$$\text{II}\quad 2\ R\text{-SH} + 2\ X\text{-}S \rightarrow 2\ RS\cdot + 2\ X\text{SH}$$

$$2\ RS\cdot \rightarrow R\text{-}S\text{-}S\text{-}R$$

In this scheme *R*-SH represents the SH-group of the enzyme and $XS\cdot$ the omeprazole radical. Reaction I shows the formation of an intermolecular disulphide accompanied by an intramolecular radical transfer, and reaction II the formation of an intramolecular disulphide by oxidizing the enzyme SH-groups. The number of free reacting SH-groups at the K^+/H^+–ATPase was 45 μmol/g protein. On the basis of a molecular weight of 100,000 for the catalytic subunit of the K^+/H^+–ATPase [2] 4 mol SH per mol enzyme can be calculated. Glutaminase which bears 1 mol SH per mol enzyme was unaffected by the omeprazole radical. Therefore, we suggest that the omeprazole radical interferes with the type II reaction at the K^+/H^+–ATPase, but we cannot exclude that other factors, e.g. different steric conformations at both enzymes might be responsible for the failure of the radical to affect the glutaminase.

In conclusion the experiments have shown that both degradation products formed from omeprazole have the ability to interfere, probably by different reaction pathways, with enzyme SH-groups.

The studies with omeprazole and the omeprazole-derived cyclic sulphenamide on glutaminase show that at pH 2.5 the cyclic sulphenamide is generated from the mother compound and that this product inactivates the enzyme by forming a disulphide. From these findings we suggest that this reaction also takes place inside the acidic secretory membrane of the parietal cell leading to inhibition of K^+/H^+–ATPase.

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