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The mechanism for inhibition of gastric ($H^+ + K^+$)-ATPase by omeprazole

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Omeprazole was found to inhibit the K^+-stimulated ATPase activity of the gastric ($H^+ + K^+$)-ATPase in parallel with the K^+-stimulated *p*-nitrophenylphosphatase activity and the phosphoenzyme formation. The degree of inhibition of ATPase activity was directly correlated to the amount inhibitor bound to the enzyme preparation down to about 15% of the control enzyme activity. The acid-decomposed form of omeprazole, i.e. the inhibitory form, was found to react with and bind to sulfhydryl groups within the ($H^+ + K^+$)-ATPase preparation with close to a 1:1 stoichiometry. β-Mercaptoethanol, when added beforehand and in a 10-fold excess of omeprazole, completely prevented binding of the inhibitor and its inhibition of the enzyme. In the presence of β-mercaptoethanol two different reaction products could be detected in addition to omeprazole; the reduced form of omeprazole (H 168/22), and a product formed between β-mercaptoethanol and a decomposition product, generated from omeprazole. Under those conditions neither inhibition nor binding was obtained, indicating that none of these three compounds was the inhibitor. Rather, the compound generated from omeprazole and reacting rapidly with either β-mercaptoethanol or the -SH groups of the enzyme was the likely inhibitor compound. In order to reverse already established inhibition higher concentrations of β-mercaptoethanol were needed than for protection indicating two different reaction pathways for protection and reversal by β-mercaptoethanol. The reversal reaction was explained by a two-step reaction; in the first step the bound inhibitor was exchanged for a β-mercaptoethanol molecule resulting in formation of compound H 168/22 and a mixed disulfide between the enzyme and β-mercaptoethanol. In the second step, attack of another β-mercaptoethanol molecule results in liberation of active enzyme and generation of the disulfide form of β-mercaptoethanol. This hypothesis was substantiated by the fact that when 1 mM β-mercaptoethanol was added to inhibited enzyme the radiolabel was partially displaced, without any change in the concentration of modified -SH groups.

Introduction

The substituted benzimidazole omeprazole has recently been shown to be a potent anti-ulcer agent [1]. Inhibition of gastric hydrochloric acid secretion by omeprazole and a related benzimidazole, picoprazole, have been demonstrated in vivo in man, the dog and the rat [2–4]. In in vitro preparations from gastric mucosa omeprazole was

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Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATP, adenosine 5′-triphosphate; ($H^+ + K^+$)-ATPase, magnesium-dependent, hydrogen ion transporting and potassium-stimulated adenosinetriphosphatase (EC 3.6.1.3).

found to effectively antagonize acid formation [5–8]. Based on these data the blockade of gastric acid secretion induced by substituted benzimidazoles has been postulated to proceed through inhibition of the gastric $(H^+ + K^+)$-ATPase [9]. This enzyme has been shown to be responsible for gastric acid secretion [10–13].

The selectivity of omeprazole as to its action in the parietal cell can be accounted for by several factors. Firstly, omeprazole accumulates into acid spaces due to its properties of being a lipid-permeable weak base. Secondly, omeprazole has been shown to be transformed into an active inhibitor of the $(H^+ + K^+)$-ATPase within the acid compartments of the parietal cell. Since the gastric $(H^+ + K^+)$-ATPase is located in the membranes separating the acid compartments and the cell cytosol, the active compound is formed close to the $(H^+ + K^+)$-ATPase in the parietal cell [14].

The aim of the present paper was to investigate the mechanism whereby omeprazole inhibits the gastric $(H^+ + K^+)$-ATPase.

Experimental procedures

Materials

Purification of $(H^+ + K^+)$-ATPase. Gastric vesicles containing $(H^+ + K^+)$-ATPase were isolated from hog stomachs in accordance with previously published methods [15].

Reagents. Na_2ATP was purchased from Sigma, U.S.A. [γ-^{32}P]ATP was obtained from Amersham International, U.K. Omeprazole, H 168/68, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl]-sulfinyl]-1*H*-benzimidazole, [^{14}C]omeprazole, and [^{3}H]omeprazole were synthesized by AB Hässle, Mölndal, Sweden. All other reagents were of the highest purity available.

Methods

Enzyme assays

$(H^+ + K^+)$-ATPase activity. The assay medium consisted of 2 mM Na_2ATP, 2 mM $MgCl_2$, 10 mM KCl, 16 mM Pipes/Tris (pH 7.4). The inorganic phosphate was analyzed according to Yoda and Hokin [16] or Lebel et al. [17]. The basal Mg^{2+}-stimulated ATPase activity, i.e. the activity found in the absence of added K^+, was found to be unaffected by omeprazole. All results concerning the enzyme activity are presented as the activity found in the presence of added K^+ minus the basal Mg^{2+}-stimulated activity.

p-Nitrophenylphosphatase activity. The assay consisted of 6 mM *p*-nitrophenyl phosphate, 6 mM $MgCl_2$, with or without 20 mM KCl, 16 mM Pipes/Tris (pH 7.4). The amount of *p*-nitrophenol released was determined according to Torriani [18].

Phosphoenzyme levels. The rate of formation and decomposition of this phosphoprotein has been shown to be faster than the overall hydrolysis of ATP. Furthermore, the formation of the phosphoprotein is ouabain insensitive and is K^+-sensitive [23,19]. Thus, by several observations, this phosphoprotein fulfils the criteria for a phosphoenzyme intermediate of the catalytic cycle.

The assay medium consisted of 5 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$ and 16 mM Pipes/Tris (pH 7.4). The amount of phosphoenzyme was determined according to Ref. 20. Blanks were prepared by adding quench solution, which contained of 10% $HClO_4$, 5 mM ATP and 40 mM inorganic phosphate to the samples before phosphorylation was initiated by [γ-^{32}P]ATP.

Protein determination

Protein was determined according to Lowry et al. [20] or Bradford [21]. Bovine serum albumin was used as standard for both methods. The Lowry method of protein determination was used for experiments in Table I and IC_{50} determinations for ATPase, *p*-nitrophenylphosphatase and phosphoenzyme levels.

Determination of omeprazole and its reaction products

Analysis was performed by means of HPLC on a 4×150 mm RP8 column with a mobile phase containing 65% phosphate buffer pH 7.6 (ionic strength = 0.025) and 35% acetonitrile. The injection volume was 100 μl and the detector operated at 280 nm. Peak areas were evaluated by an electronic integrator. For the experiments, where radiolabelled enzyme was extracted in organic solvent (Table II), the following analytical system was used; high performance liquid chromatograph constructed from an Altex 110 A constant flow

pump, a Rheodyne valve 7120 injector fitted with a home-made loop (250 μl) and an absorbance detector operated at 280 nm. The column was precisionbore stainless steel, length 150 mm. Support: Lichrosorb SI 60 (5 μm). Mobile phase: methanol, containing 5% NH_4OH (v/v)/*n*-hexane/dichloromethane (2.5 : 10 : 87,5, v/v). The flow rate was 1 ml/min.

Determination of binding levels

Separation of the membrane protein from the reaction mixture was achieved by gel filtration at room temperature over Sephadex® G-25 M (prepacked PD-10 columns, Pharmacia). The columns were washed with 25 ml H_2O and equilibrated with 25 ml 2 mM Pipes/Tris (pH 7.4) containing 70 μg albumin per ml. As described in the respective figure legend, a sample of 2.5 or 2.8 ml, respectively, was added to the column and eluted with the same solution as used for equilibration. The first 2-ml eluate was discarded, while the following 3-ml eluate was used for the assays.

Titration of sulfhydryl groups

Excess omeprazole was removed by centrifugation of the samples for 2 h at 200000 × g. The resulting pellets were rinsed with 10 ml 2 mM Pipes/Tris (pH 7.4) and resuspended by homogenization in 1 ml rinsing solution. The total amount of sulfhydryl groups in the pellets were assayed in the presence of 2% SDS by the use of DTNB as described by Habeeb [22].

Results

Effect of omeprazole on ($H^+ + K^+$)-ATPase activity, phosphoenzyme formation and K^+-stimulated p-nitrophenylphosphatase activity

During hydrolysis of ATP, catalyzed by the ($H^+ + K^+$)-ATPase, a phosphoenzyme intermediate is formed [19,23]. In addition to hydrolysis of ATP the enzyme also catalyses hydrolysis of *p*-nitrophenyl phosphate [15]. The effect of omeprazole on these three enzyme activities was studied in an incubation medium containing 50 μg of membrane protein/ml, 2 mM Pipes/Tris buffer (pH 6.0) and concentrations of omeprazole ranging between 0.1 and 100 μM for 30 min at 37°C. The inhibitory reaction was stopped by elevating the pH to 7.4 which prevented further degradation of omeprazole and, accordingly, generation of the active compound. Subsequently, the enzyme was assayed for the different enzyme activities. The control enzyme activities in the absence of omeprazole were 81 μmol/mg per h for the ATPase activity, 52 μmol/mg per h for the *p*-nitrophenylphosphatase activity and the phosphoenzyme level was 0.72 nmol/mg. All three reactions were inhibited by omeprazole with IC_{50} values being 7.8 ± 0.6 μM for inhibition of the ATPase activity, 8.5 ± 1.1 μM for inhibition of the *p*-nitrophenylphosphatase activities, 19.1 ± 0.5 μM for inhibition of the phosphoenzyme level, respectively. Values are mean ± S.E. for three determinations. Thus, both the ATPase and *p*-nitrophenylphosphatase activities were inhibited in parallel. However, the concentration needed for half maximal inhibition of phosphoenzyme formation was more than 2-times higher than the corresponding values obtained for the inhibition of the ATPase and the *p*-nitrophenyl-phosphatase activities.

Relationship between inhibition of the ($H^+ + K^+$)-ATPase activity and binding of the inhibitor

In order to investigate whether the inhibition obtained by omeprazole was reversible by washing, the K^+-stimulated ATPase activity was measured before and after washing of the membranes in omeprazole-free medium. The K^+-stimulated activity before washing was 7.9 and 95 μmol/mg per h for omeprazole-treated and control preparations, respectively. After centrifugation and subsequent washing, the activities were 7.6 and 87 μmol/mg per h for the omeprazole-treated and control preparations, respectively. Thus, the ATPase activity of the omeprazole-treated ($H^+ + K^+$)-ATPase preparation was not reversible by washing.

The time-courses for inhibition of ATPase activity and binding were followed at different concentrations of omeprazole, Fig. 1. Increasing concentrations of omeprazole enhanced the level of bound ^3H-label with time. In parallel, the enzyme activity was decreased. In Fig. 2 the remaining enzyme activity for the indicated time points and concentrations of omeprazole obtained in Fig. 1 was plotted versus the corresponding binding levels. Regardless of the concentrations of

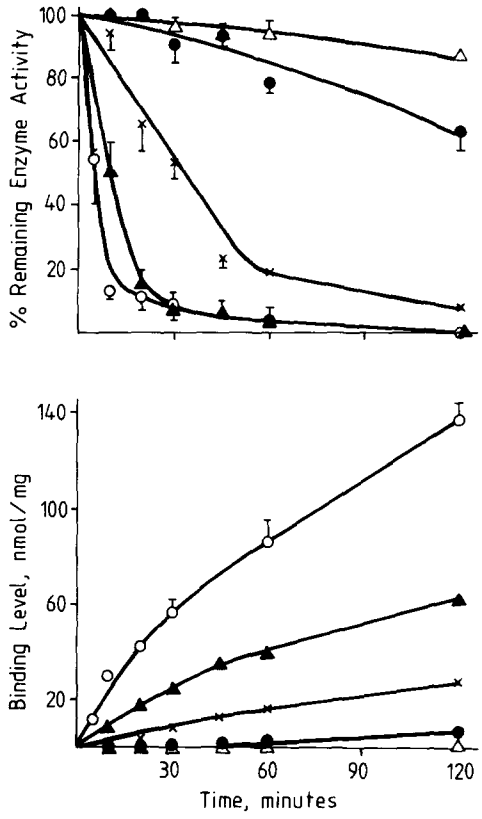

Fig. 1. Time-course for inhibition of and binding to $(H^+ + K^+)$-ATPase in the presence of omeprazole. 50 μg/ml membrane protein was incubated with [^{3}H]omeprazole (△, 1 μM; ●, 3 μM; ×, 10 μM; ▲, 30 μM; ○, 100 μM) in 4 mM Pipes/Tris (pH 6.0). Aliquots of 2.5 ml was removed at the times indicated and 300 μl 0.4 M Pipes/Tris (pH 7.4) containing 70 μg/ml albumin was added in order to stop the reaction. Excess inhibitor was removed by gel filtration and the $(H^+ + K^+)$-ATPase activity (upper figure), and binding levels (lower figure) were determined.

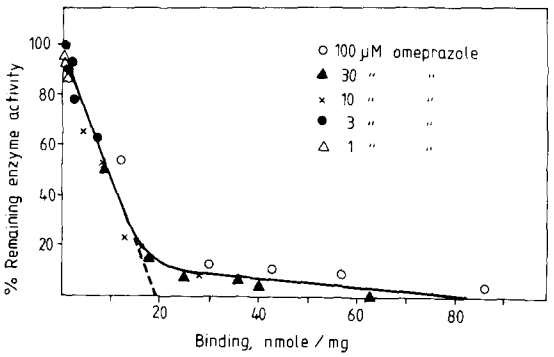

Fig. 2. Correlation between inhibition and binding. Data were taken from Fig. 1.

omeprazole and incubation times used, the inhibitory degree, down to about 15% of the control activity, was found to directly correlate to the amount of bound radiolabel. Total inhibition of the enzyme activity was obtained at a binding level of 20 nmol/mg. However, even after 85% inhibition of the ATPase activity was obtained, binding of radiolabelled compound into the preparation continued, indicating that additional binding sites were available for the inhibitor.

Role of sulfhydryl groups in the inhibitory mechanism of omeprazole

Previous investigations have revealed that mercaptans, such as β-mercaptoethanol, were able to reverse inhibition of acid formation and ATPase activity established by omeprazole, both in isolated gastric glands and partly purified $(H^+ + K^+)$-ATPase preparations, respectively [14].

In Table I, the effect of β-mercaptoethanol on both binding and inhibition of the $(H^+ + K^+)$-ATPase is shown. In this experiment β-mercaptoethanol was premixed with the enzyme before addition of [^{14}C]omeprazole. It follows that both binding and inhibition was effectively prevented in the presence of β-mercaptoethanol. In

TABLE I

PROTECTION BY β-MERCAPTOETHANOL AGAINST INHIBITION OF AND BINDING TO $(H^+ + K^+)$-ATPase INDUCED BY OMEPRAZOLE

50 μg/ml protein in 2 mM Pipes/Tris (pH 6.1) was incubated 60 min with 10 μM [^{14}C]omeprazole with or without 100 μM β-mercaptoethanol as outlined above. The reaction was stopped by elevation of pH to 7.4. Excess omeprazole was removed by sedimentation of the membranes (200000 × g for 120 min), and the protein content, binding levels and K^+-ATPase activity were determined as described in Methods.

Conditions	$(H^+ + K^+)$-ATPase activity (μmol/mg per h)	Binding level (nmol/mg)
1. Enzyme + β-mercaptoethanol (pH 6.1)	88	–
2. Enzyme + omeprazole (pH 6.1)	8 ± 1	16 ± 0.6
3. Enzyme + β-mercaptoethanol + omeprazole (pH 6.1)	95 ± 10	0.7 ± 0.1

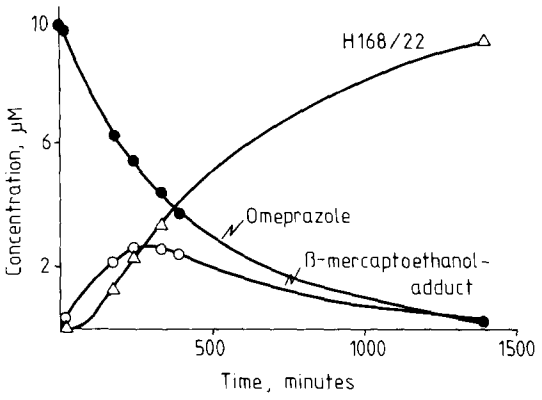

Fig. 3. Decomposition of omeprazole in the presence of β-mercaptoethanol. 10 μM omeprazole was incubated in 4 mM acetic acid/Tris (pH 6.21) with 0.1 mM β-mercaptoethanol for various periods of time. Analysis of the reaction compounds was performed as described under Methods.

Fig. 3, the concentrations of omeprazole and its reaction products were followed in the presence of β-mercaptoethanol under conditions as described in Table I, except that no enzyme was present, which did not influence the degradation of omeprazole or the product formation pattern. In addition to omeprazole, two compounds were identified; the reduced form of omeprazole (H 168/22), and the adduct between β-mercaptoethanol and a decomposition product of omeprazole. In a parallel experiment performed at pH 4.0, the formation of compound H 168/22 was found to closely follow the appearance of the disulfid form of β-mercaptoethanol (data not shown). The effect of β-mercaptoethanol on the decomposition rate of omeprazole was investigated at pH 5.26 and 37°C in an 4 mM acetic acid/Tris buffer containing 10 μM omeprazole with and without 10 mM β-mercaptoethanol. The decomposition of omeprazole was in both cases found to follow a first-order reaction mechanism. The rate constant for degradation of omeprazole was $2.17 \cdot 10^{-2} \pm 2.1 \cdot 10^{-4}$ (S.D.) min^{-1} in the absence and $2.01 \cdot 10^{-2} \pm 0.22 \cdot 10^{-4}$ min^{-1} in the presence of 10 mM β-mercaptoethanol. Thus, since the decomposition of omeprazole was found to proceed through the same mechanisms and with the similar rate constants, irrespective of whether β-mercaptoethanol was present or not, it can be concluded that there is no direct interaction between omeprazole and β-mercaptoethanol.

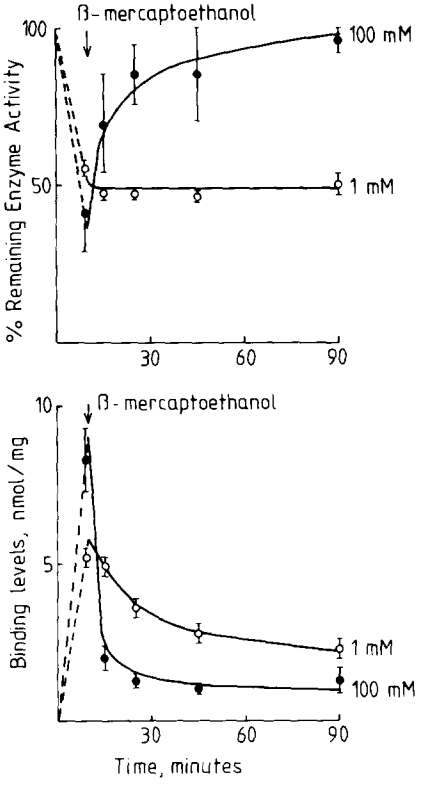

Fig. 4. Reversal of omeprazole-induced inhibition of $(H^+ + K^+)$-ATPase by β-mercaptoethanol. Upper figure shows the effect on the K^+-ATPase activity and the lower the effect on binding. 50 μg/ml membrane protein in 4 mM Pipes/Tris (pH 6.0) was incubated with 30 μM [^{3}H]omeprazole at 37°C for 10 min and subsequently 100 mM or 1 mM β-mercaptoethanol in 30 mM Pipes/Tris (pH 7.4) was added. At the times indicated aliquots of 2.5 ml were removed and the enzyme preparation was separated from the reaction mixture by gel filtration.

Displacement of the bound ^{3}H-radiolabel from the $(H^+ + K^+)$-ATPase preparation, following addition of β-mercaptoethanol, is shown in Fig. 4. In this experiment inhibition of the ATPase activity and binding to the preparation were allowed to proceed for 10 min whereafter 100 mM β-mercaptoethanol was added. The mercaptan was found to both restore the enzyme activity and displace the bound radiolabel, Fig. 4 upper and lower, respectively. In contrast, when the β-mercaptoethanol concentration was reduced to 1 mM no reactivation of the ATPase activity was obtained despite the fact that a substantial part of the bound radiolabel was released, Fig. 4. It should

also be noted that despite the fact that 1 mM β-mercaptoethanol was a too low concentration to reverse the enzyme activity, 0.1 mM β-mercaptoethanol completely protected the ($H^+ + K^+$)-ATPase from inhibition when added before omeprazole, Table I, indicating different reaction pathways for the protective–as opposed to the reversal reactions.

Titration of sulfhydryl groups in the ($H^+ + K^+$)-ATPase preparation at different binding levels are shown in Fig. 5. In this plot, where the amount of modified sulfhydryl groups were plotted against the amount of bound radiolabel, a straight line was obtained with a slope of 1.2, indicating that the inhibitor reacts with -SH groups with not far from a 1 : 1 stoichiometry. However, inclusion of 1 mM β-mercaptoethanol after the inhibitory reaction had been stopped resulted in a slope close to 2, Fig 5. This result was obtained because half of the amount of bound inhibitor had been liberated but the corresponding -SH groups were still modified, i.e. not available for titration.

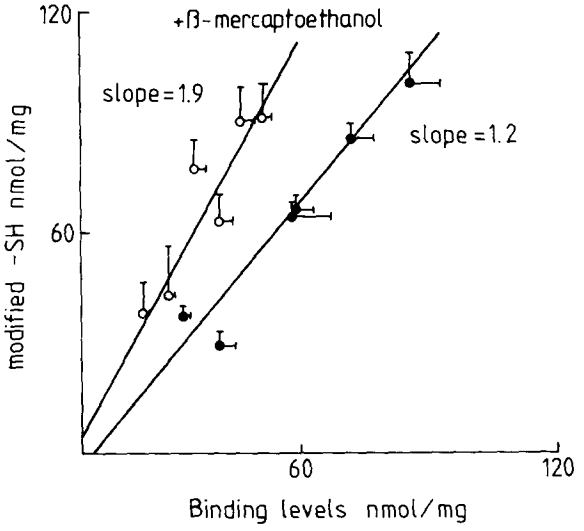

Fig. 5. Correlation between binding of inhibitor and concentration of sulfhydryl groups, 50 μg/ml enzyme preparation was incubated at 37°C in 4 mM Pipes/Tris (pH 6.0) with 30 μM [^{3}H]omeprazole. At various times ranging between 30 to 120 min the reaction was stopped by addition of Pipes/Tris (pH 7.4) to a final concentration of 43 mM. The preparation was made free from excess omeprazole by centrifugation and the concentration of total -SH groups was determined as described in Methods. The amount of modified sulfhydryl groups was calculated as the difference between the total amount present in control minus the total amount in omeprazole reacted enzyme. 1 mM β-mercaptoethanol was added after the inhibitory reaction had been terminated. Incubation with the mercaptane was allowed to proceed for 15 min at 37°C and subsequently 15 min at room temperature before the centrifugation was started. ●, in absence of β-mercaptoethanol; ○, in presence of 1 mM β-mercaptoethanol.

In an attempt to identify the ^{14}C-radiolabelled compound released from the enzyme preparation, following β-mercaptoethanol treatment, organic solvent extraction was performed, Table II. Extraction performed in the presence of β-mercaptoethanol and methylene chloride gave the highest yield, with about 83% of the total radioactivity recovered in the organic phase. The remaining 17% was either found in the water phase and/or still bound to the preparation which sedimented in the interphase. In contrast, when extraction was performed in the absence of β-mercaptoethanol only about 33% of the total radiolabel was found in the organic phase. Table II. The radiolabelled material recovered in the organic phase after β-mercaptoethanol treatment was subjected to HPLC analysis which showed that only two radioactive species were present. The major compound present was identified as H 168/22, whereas the second compound was omeprazole.

TABLE II

EXTRACTION OF THE BOUND RADIOLABEL FROM THE ($H^+ + K^+$)-ATPase PREPARATION

After inhibition of the enzyme with 10 μM [^{14}C]omeprazole for 20 min at pH 6.1, the preparation was washed free of excess inhibitor; the samples were centrifugated at 200000 × g for 2 h and the resulting pellets were washed twice with 2 mM Pipes/Tris (pH 7.4). The final pellets were resuspended by homogenisation in the washing buffer. 50000 dpm of bound radiolabel was extracted at pH 7.0 to 7.4 for 1 min with an equal volume of CH_2Cl_2 with or without 0.14 M β-mercaptoethanol.

Extraction conditions	Total bound radioactivity before extraction (dpm)	Recovery after extraction * in the organic phase (dpm)
CH_2Cl_2	50000	16500 ± 2600
CH_2Cl_2 + 0.14 M β-mercaptoethanol	50000	41700 ± 1500

* The remaining radioactivity was found in the water phase and in the preparation, which sedimented in the interphase.

The amount of omeprazole present was less than 10% of the amount of H 168/22. It can thus be concluded that the predominant compound released from the preparation was H 168/22.

Discussion

Omeprazole was found to inhibit the K^+-stimulated ATPase activity of the $(H^+ + K^+)$-ATPase. However, since omeprazole also counteracted phosphoenzyme formation and *p*-nitrophenylphosphatase activity it is not possible to define which partial reaction(s) of the catalytic cycle that was affected.

Based on the data presented a model for the chemical reactions leading to inhibition of gastric $(H^+ + K^+)$-ATPase, and thereby acid secretion, is shown in Fig. 6. In this scheme Reaction 1 represents protonation of omeprazole. It has previously been shown that omeprazole is accumulated within the parietal cell as a function of the degree of intraglandular acidity [14]. The accumulation is explained by the impaired permeability of the charged form of omeprazole. In Reaction 2 omeprazole is transformed into the active inhibitor, I. Previous publications have revealed that acid treatment of omeprazole is a prerequisite for inhibition, since no inhibition was obtained when the isolated mucosal preparation was buffered or when the purified $(H^+ + K^+)$-ATPase was treated with omeprazole at high pH, i.e. transformation was prevented [14,6]. The conclusion that the inhibitor I is generated from omeprazole is further substantiated by the data presented in Table I where omeprazole was decomposed in the presence of the $(H^+ + K^+)$-ATPase preparation and *β*-mercaptoethanol. Under those conditions no inhibition was obtained. The chemical reactions of omeprazole were studied under similar conditions (Fig. 3) and the only compounds found in addition to omeprazole were compounds H 168/22 and the reaction product between I and *β*-mercaptoethanol (I-S-S-R). This indicates that neither of these three compounds were inhibitors of the enzyme. The lack of inhibition under those conditions are not due to reversal of the inhibitory reaction (Reaction 5, Fig. 6), since 0.1 mM *β*-mercaptoethanol could not reverse already established inhibition. The absence of inhibition is more likely explained by reaction pathway 3 (Fig. 6), where excess *β*-mercaptoethanol effectively competes with the enzyme sulfhydryl groups for the inhibitor I and thereby prevents both inhibition and binding. However, in the absence of exogenous sulfhydryl compounds, both binding to and inhibition of $(H^+ + K^+)$-ATPase occurred, represented by Reaction 4 in Fig. 6. In this reaction the acid decomposed form of omeprazole, I, was found to react with and bind to sulfhydryl groups. The inhibitor was found to bind tightly to the enzyme preparation, since the binding was resistant to washing, gel filtration and to a large extent to organic solvent extraction, as expected for a disulfid bond. However, the bound radioactivity was released upon addition of 1 mM *β*-mercaptoethanol, as shown in Reaction 5 in Fig. 6 which represents reversal of the inhibitory reaction. This probably proceeds in a two-step reaction. In the first step compound H 168/22 is released and thereby the bound radioactivity. However, when *β*-mercaptoethanol was added to the preformed enzyme-inhibitor complex, the amount of modified sulfhydryl groups was not changed. This indicates that *β*-mercaptoethanol is capable to exchange for the bound radiolabel. When the reversal reaction was performed at high *β*-mercaptoethanol concentration, 100 mM, restoration of the enzyme activity was obtained, presented by the second step of Reaction 5 in Fig. 6.

In vivo, inhibition of gastric acid secretion by omeprazole has been found to persist well after decline of its plasma concentration. This may be explained by the tight binding of the active compound to the $(H^+ + K^+)$-ATPase. Presently it is not known whether recovery of acid secretion in-

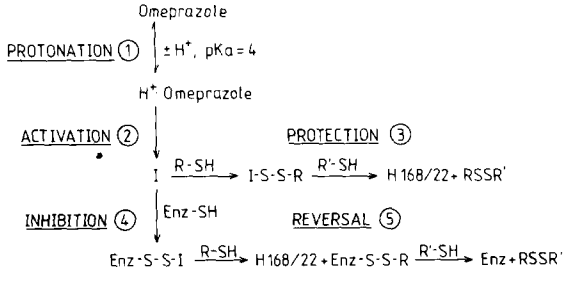

Fig. 6. Mechanism of inhibition of $(H^+ + K^+)$-ATPase by omeprazole.

volves reactivation of the $(H^+ + K^+)$-ATPase by endogenous sulfhydryl compounds or whether de novo synthesis of the enzyme is required.

Note added in proof (Received June 3rd, 1985)

When the manuscript was being printed, an alternative mechanism for inhibition of the $(H^+ + K^+)$-ATPase by omeprazole was published, Im et al. [24]. In contrast to the disulfide linkage between the enzyme and the inhibitor being the primary inhibitory complex as proposed in this paper, Im et al. suggest that the sulfhydryl groups of the enzyme initially react to give the formation of an *N*-sulfenylated complex rather than a disulfide complex.

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