Mechanism of Anti-urease Action by the Anti-ulcer Drug Ecabet Sodium

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To investigate the mechanism of the anti-urease action of ecabet sodium (ecabet) observed in Helicobacter pylori in vitro, the effects of ecabet on purified urease from jack bean were studied in comparison with the effects of the specific urease inhibitor benzohydroxamic acid (BHA). After incubation of the enzyme with the test drug for a period of time, urease activity was measured. Ecabet depressed the activity below pH 5, and the lower the pH, the greater the degree of depression. The degree of depression by ecabet increased gradually during incubation and reached a plateau in 20 min, whereas that by BHA attained a maximum rapidly. The IC_{50} values of ecabet and BHA were 2.1 mg/ml and 0.5 µg/ml, respectively. When the incubation mixture of urease with an inhibitor was diluted and further incubated, the depressed activity by BHA reverted gradually, but that by ecabet did not. When the incubation mixture of urease with ecabet was centrifuged, the urease activity of the mixture decreased in parallel with the reduction in concentration of the supernatant. When the incubation mixture of urease and ^14C-ecabet was ultrafiltered to remove the drug, the radioactivity in the retentate remained in parallel with the degree of reduction of urease activity in the retentate. These results indicate that ecabet irreversibly depresses the urease activity of jack bean, and suggest that the depression is caused by irreversible binding of ecabet to urease followed by denaturation of the enzyme protein.

Key words ecabet sodium; anti-ulcer drug; benzohydroxamic acid; anti-urease action; jack bean urease

Helicobacter pylori, a spiral-shaped gram-negative bacterium which is frequently isolated from gastric biopsies of patients with gastritis and gastroduodenal ulcer, is now recognized as playing a role in the pathogenesis of gastroduodenal disorders.1-4) H. pylori is characterized by very high urease activity.5-7) Urease generates ammonia in the immediate bacterial microenvironment to protect the organism from the deleterious effects of gastric acid.8) Ammonia is known to alter gastric mucosal structures and functions9) and to be toxic to eukaryotic cells.10,11) Thus, the urease activity of H. pylori acts as a survival factor for the organism and a virulence factor for the host.

Ecabet sodium (ecabet) is a locally acting anti-ulcer drug12-14) which possesses an anti-peptic action13,15) and gastroprotective effects.13,16) We have recently observed that ecabet depresses the urease activity of H. pylori and interferes with the survival of the organism at an acidic pH in vitro.17) Thus, ecabet may be efficacious in H. pylori-related gastrointestinal diseases through its anti-urease action.

H. pylori urease has some characteristics in common with jack bean urease, while each enzyme differs in the number of subunits18) and optimum pH.19,20) Namely, the amino acid composition of urease is highly conserved between the plant and bacterial species,6,18) and both ureases are inhibited by common inhibitors, such as hydroxamic acid derivatives.5) Jack bean urease can probably be used as a model to study the mechanism of the anti-urease action of ecabet in H. pylori. In the present study, we investigated the effects of ecabet on a purified preparation of urease from jack bean in comparison with those of a specific urease inhibitor, benzohydroxamic acid (BHA).21) In addition, the binding of ecabet to urease was studied.

MATERIALS AND METHODS

Compounds and Reagents The disodium salt of ecabet was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd. BHA and purified urease from jack bean (95000 units/g protein) were purchased from Sigma (St. Louis, MO, U.S.A.). ^14C-ecabet was synthesized in Amersham International plc (Buckinghamshire, U.K.). The radiochemical purity was more than 98% and the specific activity was 3.5 MBq/mg. The labeled compound was diluted with unlabeled ecabet to various degrees depending on the experimental conditions. Urea was purchased from Nakalai Tesque (Kyoto, Japan) and dissolved in purified water. The micro BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) was used for the measurement of protein concentration.

Effects on Urease Activity 1) Assay of Urease Activity: Urea (0.05 ml, 200 mM) was added to the enzyme solution (0.95 ml) to start the reaction. The reaction mixture was incubated at 37°C for 1 min, and then mixed with 1 N sulfuric acid (0.25 ml) to stop the reaction. The ammonia produced was determined by the indophenol method,22) and urease activity was expressed as mmol NH_3/min/mg of the enzyme.

2) Experimental Protocols: Urease was dissolved in 50 mM citrate buffer at various pH values. Unless otherwise stated, the final concentration of the enzyme was 50 µg/ml. Three series of experiments were carried out as follows.

Protocol 1: a mixture (0.95 ml) of urease and test drug was preincubated at 37°C for a period of time, and then urease activity was measured.

Protocol 2: a mixture of urease (100 µg/ml) and test drug was preincubated for 10 min, then diluted 100-fold with the buffer, and further incubated for a period of time. An aliquot (0.95 ml) of the diluted solution was assayed for urease activity.

Protocol 3: a mixture (1.9 ml) of urease and test drug

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was incubated for 10 min, and half of it (0.95 ml) was used to measure the enzyme activity. The remaining half was centrifuged at 1870 \( \times g \) at 4°C for 10 min, and then the protein concentration of the supernatant was measured.

Enzyme activity and protein concentration were measured in duplicate, and those without the test drug were used as a control to calculate the percent inhibition.

**Binding to Urease** To estimate the binding of ecabet to urease, an ultrafiltration method was performed as described below. Since jack bean urease consists of six identical 90.8 kDa subunits,\(^{19}\) centircen-50 (molecular weight cut off < 50000, Amicon, MA, U.S.A.) was used for ultrafiltration. The solution containing urease (1.9 ml, 50 \( \mu \)g/ml final) was mixed with \(^{14}\)C-ecabet (0.1 ml) and incubated at 37°C for 20 min. The whole incubation mixture was placed into the ultrafiltration apparatus and centrifuged (1000 \( \times g \), 20°C) for 9 min to concentrate it to about 0.5 ml. The retentate was diluted to 2 ml using the buffer. This process was repeated 12 times, then radioactivity in the retentate was measured by liquid scintillation counter (Packard Tri-Carb 460CD, IL, U.S.A.).

The same procedure was performed using unlabeled ecabet. Urease activity and protein concentration in the retentate were measured.

Ultrafiltration was carried out in triplicate.

**RESULTS**

**Effects of Ecabet on Urease Activity** The activity of jack bean urease was observed in a broad pH range of 4.0—8.0 with a pH optimum of 6.0. Its pH-activity curve was shallowly convex (data not shown). Ecabet at 4 mg/ml depressed the urease activity below pH 5. The degree of depression by the drug became stronger as the pH decreased (Fig. 1). On the other hand, BHA at 10 \( \mu \)g/ml showed almost constant degrees of inhibition in the pH range of 4.4—5.0 (Fig. 1).

As shown in Fig. 2, depression by ecabet at 4 mg/ml increased gradually depending on the duration of preincubation of the enzyme with the drug; 10 min of preincubation was needed for more than 90% depression. On the other hand, BHA at 10 \( \mu \)g/ml inhibited the urease activity over 90% after only 1 min of preincubation.

After 20 min of preincubation, ecabet at 1—4 mg/ml and BHA at 0.1—10 \( \mu \)g/ml concentration-dependently depressed the urease activity (Fig. 3); the IC\(_{50}\) values of the two drugs were 2.1 mg/ml and 0.5 \( \mu \)g/ml, respectively. Such concentration-dependency of the anti-urease action of both drugs was independent of the enzyme concentration between 0.5 to 100 \( \mu \)g/ml (data not shown).

When the concentration of BHA was reduced from 10 to 0.1 \( \mu \)g/ml by 100-fold dilution and the diluted mixture was further incubated (protocol 2), the percent inhibition by BHA at 10 \( \mu \)g/ml of the undiluted mixture (ca. 90%) decreased over time to ca. 20%, which was almost the...
same degree as that caused by the drug added at the concentration of 0.1 μg/ml (Figs. 3, 4). On the other hand, the degree of depression caused by ecabat did not change with time after the dilution followed by incubation (Fig. 4).

Although the incubation mixture of ecabat (1—4 mg/ml) and urease (protocol 3) produced no apparent precipitation, when it was centrifuged, the protein concentration of the supernatant decreased depending on the drug concentration (Fig. 5). The percent reduction of urease activity in the mixture was parallel with the degree of reduction of protein concentration in the supernatant (Fig. 5).

**Binding of Ecabat to Urease** After the ultrafiltration of 14C-ecabat alone under these experimental conditions, radioactivity in the retentate was not detected. When the incubation mixture of 14C-ecabat with urease was ultrafiltered, the radioactivity in the retentate was observed depending on the drug concentration of the incubation mixture, and was parallel with the degree of reduction of urease activity in the retentate (Fig. 6).

**DISCUSSION**

In the present study, ecabat depressed the urease activity of jack bean in a pH-dependent manner; the lower the pH, the greater the inhibition. Ecabat has a sulfonic and a carboxyl group, and the percentage of the undissociated form of the drug became higher as the pH decreased.23) The hydrophobicity of ecabat increased depending on the percentage of its undissociated form.23) Thus, the hydrophobic interaction between the drug and the enzyme is suggested to be involved in the inhibition of urease by ecabat. The influence of pH on the percentage of undissociated sulfonic and/or carboxyl groups of ecabat may explain the pH-dependency of its anti-urease action.

Ecabat showed the time-dependent inhibition of jack bean urease, and the depressed activity was not reversed by dilution. By contrast, the depression by BHA, a reversible urease inhibitor,25) rapidly attained the maximal constant degree, and the depressed activity reverted on dilution. Generally, reversible inhibition is characterized by a definite degree of inhibition which is usually attained rapidly.24) Reversibility of inhibition is also demonstrated by the dilution method, since dilution of an enzyme-inhibitor complex will cause some dissociation.24) The characteristics of the depression of urease activity by BHA shown in this study are those of reversible inhibition. On the other hand, the depression of urease activity by ecabat appears to be irreversible. However, it seems too hasty to conclude from these results that the effect of ecabat was caused by enzymatic inhibition.

We have already reported that ecabat reduces the peptic activity of gastric juice by precipitating pepsin.15) This observation suggests that the anti-peptic action of the drug was caused not by enzymatic inhibition but by denaturation of the enzyme protein, since aggregation or precipitation of the protein often accompanies the denaturation.25) In the present study, the protein concentration of the supernatant was shown to decrease depending on the concentration of ecabat after centrifugation of the incubation mixture of urease with the drug. Furthermore, the decrease in protein concentration was parallel with the reduction of urease activity in the incubation mixture. These results indicate that ecabat depressed the urease activity by aggregating the enzyme. Thus, it is suggested that the anti-urease action of ecabat, as well as its anti-peptic action, is based on denaturation of the enzyme protein.

After ultrafiltration of the solution containing 14C-ecabat alone, no radioactivity in the retentate was detected, indicating that the ultrafiltration procedure used in the present study removed any non-bound drug from the system. Therefore, radioactivity in the retentate obtained by ultrafiltration of the incubation mixture of 14C-ecabat with urease seems to reflect the amount of the drug bound to the urease. Depression of urease activity in the retentate was parallel with the amount of the bound drug, suggesting that this binding is responsible for its anti-urease action.

If ecabat reversibly and rapidly interacts with the urease, reduction of the free drug concentration by ultrafiltration would cause some dissociation of the drug-enzyme complex during centrifugation and/or sampling time, and also, after the repetitive ultrafiltration, bound ecabat would not be detected. However, we found that the binding of ecabat to urease was preserved after the repetitive ultrafiltration, suggesting that ecabat is irreversibly bound to urease. We
have already reported that radioactivity could not be completely removed from the incubation mixture of $^{14}$C-ecabet with jack bean urease after 72 h of dialysis, which seems to support the irreversibility of the binding. The irreversible feature of the binding of ecabet to urease is probably reflected in the irreversibility of its anti-urease action.

In conclusion, ecabet irreversibly depressed the urease activity of jack bean, and the depression is probably caused by denaturation of the enzyme through irreversible binding of ecabet to urease. Since the inhibition of urease by ecabet in \textit{H. pylori} was also time- and pH-dependent, ecabet probably depressed the urease activity of this organism by denaturation of the enzyme protein.

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REFERENCES