Volume 299, number 1, 51–53 FEBS 10782 March 1992

Aziridine-2-carboxylic acid

A reactive amino acid unit for a new class of cysteine proteinase inhibitors

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Received 11 December 1991; revised version received 17 January 1992

The three-membered ring of aziridine-2-carboxylic acid, which is susceptible to opening by nucleophiles, has been analyzed as a potential useful handle for the design of specific irreversible inhibitors of cysteine proteinases. For this thiol-reactive amino acid, an imino analogue of proline, a second-order rate constant of 17.07 $M^{-1}\cdot s^{-1}$ for inactivation of papain was determined. Thus, the aziridine moiety proved to be remarkably more reactive than activated double bonds, e.g. *N*-ethylmaleimide, or halides such as α-iodopropionic acid or chloroacetic acid. Since it does not alkylate histidine under conditions in which quantitative alkylation occurs with *N*-ethyl-maleimide, it could represent an interesting reactive amino acid unit for the synthesis of a new class of irreversible inhibitors, at least in terms of specificity of the chemical reaction involved in the inactivation process.

Aziridine-2-carboxylic acid; Thiol reactivity; Irreversible inhibitor; Cysteine proteinase; Papain

1. INTRODUCTION

Affinity labelling of the active-site cysteine residue of cysteine proteinases has led to the successful design of irreversible inhibitors needed for clarifying the role of these enzymes in disease processes. Among the various approaches used for such purpose [1,2] the specific alkylation of the active-site thiol function represents a reaction principle extensively exploited for this purpose. Correspondingly, a series of suitable thiol-reactive groups has been developed and incorporated into substrates in order to build-in enzyme specificity. Of the alkylating agents examined in this context the oxiran ring of the *trans*-epoxysuccinyl moiety, present in the natural inhibitor E-64, allowed the production of a series of powerful active-site-directed inhibitors [1–3].

The three-membered aziridine ring (Fig. 1), which is also known to react with nucleophiles, is structurally similar to oxiran, and, depending upon N- and C-substituents, it is opened more or less rapidly and selectively by thiols [4,5]. This thiol-alkylating property of aziridine derivatives, however, has been exploited only in a limited manner for the development of thiol-specific reagents useful for protein modifications [6]. Among the known aziridine compounds, aziridine-2-carboxylic acid, an imino analogue of proline, can be prepared from suitably protected serine derivatives [7,8] and a priori this amino acid unit could represent an interesting and highly promising reactive handle for the development of a new class of active-site-directed inhibitors of cysteine proteinases.

In the present communication we report on the inactivation of papain, as the best known example of cysteine proteinases, by aziridine-2-carboxylic acid as the thiol-trapping reagent without built-in enzyme specificity.

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2. EXPERIMENTAL

2.1. *Materials*

Papain (BioChemika, powder, 10.7 U/mg) was from Fluka; Z-Gly-ONp was prepared by standard procedures and recrystallized from 90% aqueous ethanol. 4-(4-Nitrobenzyl)-pyridine was purchased from Fluka and recrystallized from acetone–cyclohexane. Aziridine-2-carboxylic acid and its amide were synthesized following the route described by Nakajima et al. [7]; the analytical data of the resulting compound were in good agreement with the literature values [8] and the ^{1}HNMR data were consistent with the assigned structures.

2.2. *Enzyme assays*

Solutions of papain were prepared fresh, daily, by incubating the enzyme (10^{-6} M) in 0.05 M sodium phosphate (pH 6.5), 5 mM EDTA with 5×10^{-4} M cysteine for 45 min at 25°C.

Assays were performed at 4.0×10^{-7} M concentration of papain in the 0.05 M phosphate buffer (pH 6.5) containing 5 mM EDTA 2.1×10^{-4} M cysteine and Z-Gly-ONp (10^{-4} M). The rate of the enzymatic hydrolysis of the substrate at 22°C was monitored continuously at 400 nm against the non-enzymic hydrolysis of the substrate induced by the presence of cysteine. Substrate and inhibitor were dissolved in DMF, the final concentration of which was 16.6% v/v.

For the inhibition experiments papain (4.5×10^{-7} M) in 0.05 M phosphate (pH 6.5), 5 mM EDTA, 2.35×10^{-4} M cysteine was pre-incubated with aziridine-2-carboxylic acid in the concentration range of 4.5×10^{-7} to 9.0×10^{-5} M at 22°C for 5–60 min; then the substrate was added and

the residual enzyme activity at time t was determined as described above.

To confirm that irreversible inhibition of papain occurs in these experiments, the enzyme (4.5×10^{-7} M) was completely inactivated with aziridine-2-carboxylic acid (4.5×10^{-5} M for 60 min) and then subjected to gel-chromatography on Biogel P-4. The control enzyme maintained the activity after chromatography, whereas for the inactivated enzyme no activity could be recovered.

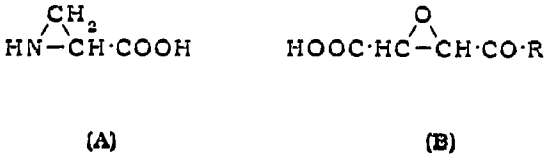
2.3. *Determination of rate constants*

Experimental data of the inactivation of papain were treated according to the model of non-specific irreversible inhibitors, i.e. in terms of a bi-molecular protein modification reaction [10] using the programme GraFit [11].

3. RESULTS AND DISCUSSION

Studies on irreversible inhibition of papain by α-iodopropionic acid [12,13] and chloroacetic acid [14] clearly revealed a bell-shaped, pH-dependent reaction rate of these thiol reagents with a maximum around pH 6.0. This behaviour was assigned to intraprotein electrostatic interactions between sulfhydryl, imidazolium and carboxylate groups causing a remarkably increased nucleophilicity of the active-site sulfur anion. Furthermore from a comparison of the reaction rates of these alkylating agents as acids and amides it was deduced that ionic interactions with the active site of the enzyme is remarkably affecting the inactivation rates of papain. These interactions were proposed to be responsible for a stereo-specific orientation of the α-iodopropionic acid onto the reaction center and thus for the 10^3-fold higher second order rate constant determined for the inactivation of papain by $L(-)$-α-iodopropionic acid ($4.9\times M^{-1}\cdot s^{-1}$) than for the reaction of this halide derivative with cysteine (3.3×10^{-3} $M^{-1}\cdot s^{-1}$) at pH 7.0 and 25°C. Similar stabilization and orientation effects by electrostatic interactions were also expected to occur for the aziridine-2-carboxylic acid as potential alkylating agent of the active-site cysteine of papain.

For measuring the inactivation potency of aziridine-2-carboxylic acid initial enzyme activities were determined at increasing papain/aziridine-2-carboxylic acid ratios (1:20 to 1:200) as a function of incubation time. Semi-logarithmic plots of the fractional enzyme activity remaining (a_t) against time (t) of incubation with aziridine-2-carboxylic were found to be linear with correlation coefficients, $r = 0.99$, thus allowing estimates of the pseudo-first-order inactivation rates (k_{obs}) at the various enzyme/inhibitor ratios (Fig. 2). Evaluation of the kinetic data according to Kitz and Wison [15] indicates a



(A) (B)

Fig. 1. Aziridine-2-carboxylic acid (A) and the oxiran moiety (B) of the natural inhibitor E-64.

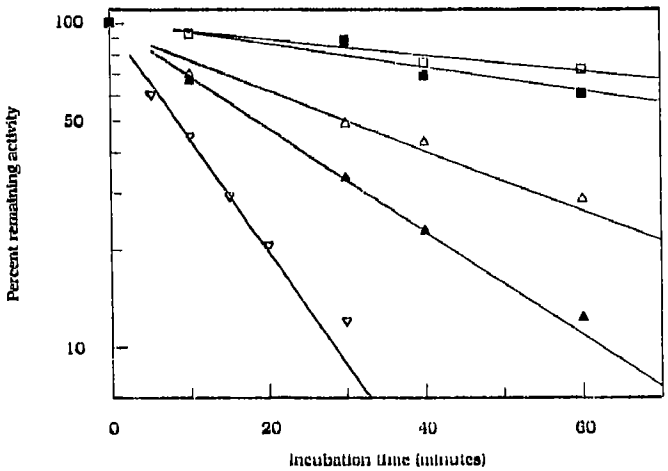


Fig. 2. Inactivation of papain with aziridine-2-carboxylic acid at increasing enzyme/inhibitor ratios: 1:20 (□), 1:30 (■), 1:50 (△), 1:100 (▲) and 1:200 (▽).

very high dissociation constant (K_i) of the enzyme–inactivator complex. In fact, the k_{obs} were found to be a linear function of the inhibitor concentration ($r = 0.998$) and a second-order rate constant (k) of 17.07 $M^{-1}\cdot s^{-1}$ was determined for the inactivation process. The experimental data are therefore consistant with the model of aziridine-2-carboxylic acid representing primarily a non-specific irreversible inhibitor with an inactivation potency which is significantly superior to that of other thiol-trapping reagents, e.g. *N*-ethylmaleimide (1 $M^{-1}\cdot s^{-1}$, at 40°C and pH 6.0 with cathepsin B) [3], $L(-)$-α- iodopropionic acid (4.9 $M^{-1}\cdot s^{-1}$ at 25°C and pH 7.0 with papain) [12] and chloroacetic acid (4.23 $M^{-1}\cdot s^{-1}$ at 30.5°C with papain) [14]. The enzyme-specificity of this thiol alkylating agent in presence of the large excess of cysteine, used as an additive in the assay medium, relies mainly on the different pK_a values of the active site and cysteine thiol group and thus on their markedly different nucleophilicity. This was confirmed in seperate experiments where we have analyzed the reaction rate of aziridine-2-carboxylic acid with an excess of cysteine under conditions of the enzyme assay (22°C, pH 6.5) by colorimetric monitoring of the aziridine-2-carboxylic acid consumption using 4-(4-nitrobenzyl)-pyridine as reagent according to the method of Epstein et al. [16]. Within the limits of error of this assay system no appreciable reaction rate was observed at pH 6.5. This fully agrees with the low content of nucleophilic sulfur anion species present at this pH value ($pK = 8.25$ for cysteine at 25°C; $I = 0.1$) and the slow reaction rate reported for this system even at higher pH values [5]. In this context the reactivity of aziridine-2-carboxylic acid is similar to that of *trans*-epoxysuccinic acid [17].

It is also worthy to note that the aziridine-2-carboxylic acid amide was found to be less potent by orders of magnitude than the free carboxylic acid at pH 6.5. Similarly lowered rate constants have previously been

reported for the inactivation of papain by α-iodopropionic acid amide [12] and chloroacetamide [18], a fact which has been interpreted in terms of loss of electrostatic interaction between the carboxyl group of the thiol reagents and the active-site imidazole function. In view of these findings even the carboxyl group of aziridine-2-carboxylic acid should contribute to the stabilization and orientation of the alkylating agent, but this electrostatic interaction is insufficient to lower the dissociation constant (K_i) to kinetically significant values.

The data obtained in this first series of experiments support the working assumption that aziridine-2-carboxylic acid could represent a promising amino acid unit for the design of a new class of cysteine proteinase inhibitors if the three-membered ring is used as the reactive handle and incorporated into peptide structures suitable for substrate-type recognition and binding by the various cysteine proteinases. Its main advantage could derive from its high thiol selectivity as compared to other thiol reagents which are known to alkylate imidazole as well as amino groups, although at lower rates than thiols. Conversely, *N*-alkylation of histidine derivatives by nucleophilic ring opening of aziridine-2-carboxylic acid did not occur at room temperature under favoured conditions, e.g. in dry dimethylformamide, where histidine compounds are quantitatively converted to N^{τ}- and N^{π}-succinimidyl derivatives via reaction with *N*-ethyl-maleimide [19].

Since the active sites of cysteine proteinases are known to contain, besides a highly reactive thiol group, a histidine residue, the development of aziridine-2-carboxylic acid-containing inhibitors could become an interesting new approach at least in terms of specificity of the chemical reaction involved in the inactivation process.

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