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A new lysine derived glyoxal inhibitor of trypsin, its properties and utilization for studying the stabilization of tetrahedral adducts by trypsin

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ABSTRACT

New trypsin inhibitors Z-Lys-COCHO and Z-Lys-H have been synthesised. K_i values for Z-Lys-COCHO, Z-Lys-COOH, Z-Lys-H and Z-Arg-COOH have been determined. The glyoxal group (–COCHO) of Z-Lys-COCHO increases binding ~300 fold compared to Z-Lys-H. The α-carboxylate of Z-Lys-COOH has no significant effect on inhibitor binding. Z-Arg-COOH is shown to bind ~2 times more tightly than Z-Lys-COOH. Both Z-Lys-^{13}COCHO and Z-Lys-CO^{13}CHO have been synthesized. Using Z-Lys-^{13}COCHO we have observed a signal at 107.4 ppm by ^{13}C NMR which is assigned to a terahedral adduct formed between the hydroxyl group of the catalytic serine residue and the ^{13}C-enriched keto-carbon of the inhibitor glyoxal group. Z-Lys-CO^{13}CHO has been used to show that in this tetrahedral adduct the glyoxal aldehyde carbon is not hydrated and has a chemical shift of 205.3 ppm. Hemiketal stabilization is similar for trypsin, chymotrypsin and subtilisin Carlsberg. For trypsin hemiketal formation is optimal at pH 7.2 but decreases at pHs 5.0 and 10.3. The effective molarity of the active site serine hydroxyl group of trypsin is shown to be 25300 M. At pH 10.3 the free glyoxal inhibitor rapidly ($t_{1/2}$=0.15 h) forms a Schiff base while at pH 7 Schiff base formation is much slower ($t_{1/2}$=23 h). Subsequently a free enol species is formed which breaks down to form an alcohol product. These reactions are prevented in the presence of trypsin and when the inhibitor is bound to trypsin it undergoes an internal Cannizzaro reaction via a C2 to C1 alkyl shift producing an α-hydroxycarboxylic acid.

1. Introduction

With peptide substrates catalysis by the serine proteases proceeds via a tetrahedral intermediate formed by the addition of the catalytic serine hydroxyl group to the peptide carbonyl carbon [1,2]. It is the formation or breakdown of this tetrahedral intermediate that is the rate limiting step in catalysis with peptide substrates. Therefore it is important to determine how transition state stabilization of the tetrahedral intermediate contributes to catalysis. Such tetrahedral intermediates do not accumulate during the enzyme catalysed hydrolysis of peptide bonds [3] and so they cannot be studied directly by techniques such as NMR. However, inhibitors which react with the hydroxyl group of the catalytic serine to form transition state analogues [4] of the tetrahedral intermediate can be used to determine how the serine proteases form and stabilize tetrahedral intermediates. Extensive ^{13}C NMR studies have been undertaken on tetrahedral adducts formed by chloromethylketone derivates of subtilisin [5–8], chymotrypsin [6,8–10] and trypsin [11–13]. However, these chloromethylketone inhibitors are irreversible inhibitors that alkylate the catalytic histidine moving the catalytic groups out of their optimal positions [14]. Therefore they may not be good mimics of the catalytic tetrahedral intermediate. Also as they are irreversible inhibitors we cannot use them to quantify inhibitor binding.

Specific peptide derived glyoxal inhibitors are formed by replacing the carboxy-terminal carboxylate group (R–COOH) of a peptide with a glyoxal group (R–COCHO). These glyoxal inhibitors have been shown to be potent reversible competitive inhibitors of chymotrypsin [15–18] and subtilisin [19,20]. Using ^{13}C NMR these glyoxal inhibitors have been shown to form tetrahedral adducts with both chymotrypsin [15,17,21] and subtilisin [19,20] which have been characterized by NMR. We have also developed a procedure whereby we replace the terminal carboxylate group (R–COOH) of a peptide with a hydrogen atom (R–H). If we assume this hydrogen atom does not make a significant contribution to binding then the binding of R–H will allow us to determine how much the peptide component contributes to binding. If we also measure the binding of an equivalent peptide carboxylate (R–COOH), aldehyde (R–CHO) or glyoxal (R–COCHO) inhibitor then we can

Abbreviations: Z, benzyloxycarbonyl; Z-Lys-H, Z-NHCH$_2$(CH$_2$)$_4$NH$_3^+$; Z-Lys-COOH, Z-NHCH((CH$_2$)$_4$NH$_3^+$)-COOH; Z-Lys-COCHO, Z-NHCH((CH$_2$)$_4$NH$_3^+$)-COCHO

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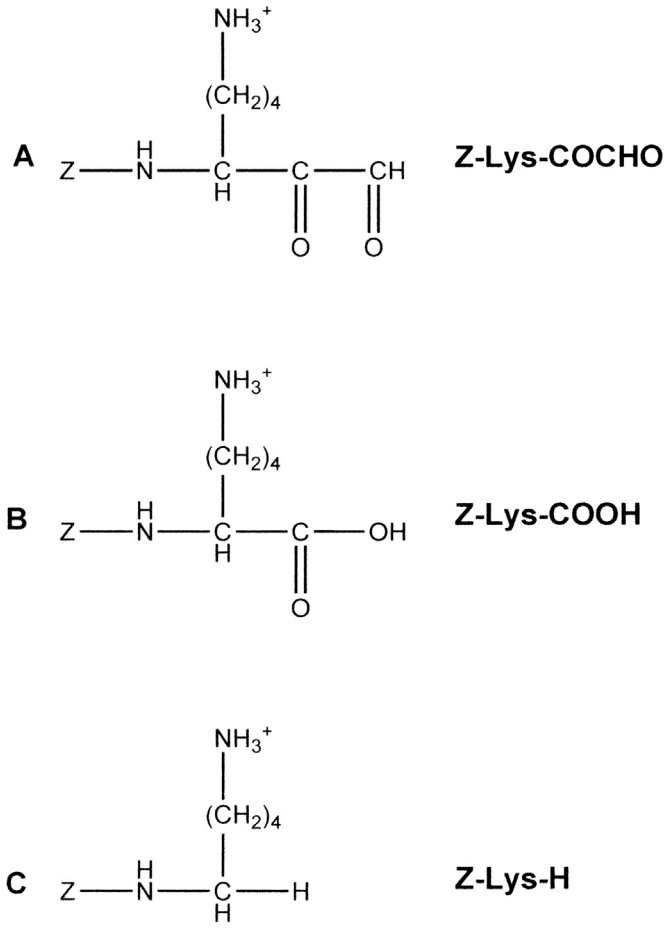


Scheme 1. Structure of Inhibitors.

quantify how the carboxylate, aldehyde and glyoxal group contribute to binding by the enzyme [22–24]. This approach has allowed us to quantify hemiketal formation in both chymotrypsin and subtilisin. The effective molarity of the catalytic serine hydroxyl group is the concentration of water required to have the same reactivity as the catalytic hydroxyl group of the serine protease. Therefore the effective molarity quantifies how the serine proteases enhance the reactivity of the catalytic serine hydroxyl group. This approach has been used to determine the effective molarity of the catalytic serine hydroxl group in both chymotrypsin and subtilisin [24]. A similar approach has been used to determine the effective molarity of the catalytic thiol group of the cysteine protease papain [23]. However, similar studies have not been carried out using specific positively charged glyoxal inhibitors of the serine protease trypsin. The synthesis of positively charged glyoxal inhibitors such as Z-Lys-glyoxal is difficult because the reactive ε-amino group must be protected during the synthesis.

In this paper we report the first synthesis of a glyoxal inhibitor (Z-Lys-COCHO) of trypsin. Z-Lys-H and the ^{13}C-enriched glyoxal inhibitors Z-Lys-^{13}COCHO and Z-Lys-CO^{13}CHO have also been synthesized. The ^{13}C-enriched inhibitors have enabled us to use ^{13}C NMR to study the stability of the inhibitor and to determine its breakdown products. We have quantified hemiketal formation with trypsin and Z-Lys-COCHO at pH 7.2 and compared it with hemiketal formation by chymotrypsin and subtilisin. This has allowed us to determine the effective molarity of the catalytic serine hydroxyl in trypsin and compare it with the effective molarity of the catalytic serine hydroxyl group in subtilisin and chymotrypsin. ^{13}C NMR with both ^{13}C-enriched glyoxal inhibitors is used to determine the structure of the Z-Lys-COCHO inhibitor when it is bound to trypsin. Finally it is shown that the enzyme catalysed breakdown of the Z-Lys-COCHO is different from its breakdown in the absence of trypsin.

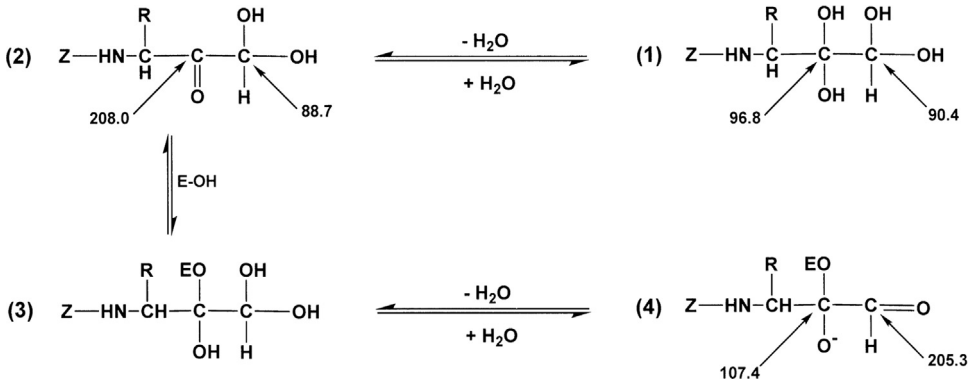
2. Materials and methods

2.1. Materials

Z-Lys(Boc) was obtained from Fluorochem Ltd, Unit 14, Graphite Way, Hadfield, Derbyshire, SK13 1QH, UK. L-[1-^{13}C]lysine:2HCl (99 at%) and [^{13}C]methylamine:HCl (99 at%) were obtained from Goss Scientific Instruments Ltd, Gresty Lane, Shavington, Crewe, Cheshire, CW2 5DD, UK. All other reagents were obtained from Sigma-Aldrich Chemical Co., Gillingham, Dorset, U.K.

2.2. Synthesis of Z-Lys-COCHO

Z-Lys(Boc) was converted to Z-Lys(Boc)-diazoketone as described by Cosgrove et al. [25] After purification by silica column chromatography the Z-Lys(Boc)-diazoketone was oxidised to Z-Lys (Boc)-glyoxal using dimethyldioxirane as described by Howe et al. [26] The t-butoxycarbonyl group(Boc) was removed by treating Z-Lys(Boc)-glyoxal in methanol with an equal volume of 1.25 M methanolic HCl for two hours at room temperature. The methanol was removed in vacuo, the product was dissolved in water and the pH adjusted to 3.0, filtered under suction and freeze-dried. The dried product was dissolved in d_6 DMSO.



Scheme 2. Reaction of Z-lys-COCHO with trypsin R=–$(CH_2)_4$–NH_3^+, EO represents the oxygen atom of the catalytic serine hydroxyl group of trypsin.