Application of Schiff Base Copper(II) and Iron(III) Chelates to Site-Specific Cleavage of a Trypsin

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Amidine-containing Schiff base iron(III) and copper(II) chelates were prepared from ω-amino acid, metal ion, and salicylaldehyde. These chelates behaved as specific inhibitors of trypsin, with Ki values in the range 10^{-5} to 10^{-6} M. Selective cleavage of the trypsin backbone resulting from specific binding of the chelate to the trypsin active site was investigated. Cleavage was observed when trypsin was incubated with amidine-containing copper(II) or iron(III) chelate, H2O2, and ascorbate. Examination of the three-dimensional structure of trypsin suggests that cleavage occurred at a peptide bond within the GLY156-ALA161 sequence.

Key words trypsin; Schiff base copper chelate; Schiff base iron chelate; synthetic inhibitor; site-specific cleavage

Site-specific cleavage of proteins and peptides promoted by redox-active metal chelates has recently been reported. Rana and Meares demonstrated that an EDTA-Fe derivative, covalently attached to a cysteine residue of the protein, can mediate cleavage of the protein backbone in a conformation-dependent manner.1-3 A different EDTA-Fe derivative attached to a protein has been described for the affinity cleavage of proteins.4-6 In these cases, cleavage of the peptide bond occurred at the region where the metal attained close contact in its three-dimensional structure. This process is more advantageous than cleavage by proteolytic enzymes or by cyanogen bromide7 for the investigation of the tertiary structure of a protein. However, the design of metal chelates which are specific and reactive to a particular protein is not a trivial matter.

In the previous paper, we reported Schiff base copper(II) chelates (1a—i, 2a—i) carrying an amidinium group that were prepared from ω-amino acids, copper(II) acetate, and salicylaldehyde, and which exhibited strong binding affinity for bovine trypsin.8 These compounds possess a cationic amidinium group which has an electrostatic interaction with the anionic carboxyl group of the ASP77 residue9 of the trypsin binding cavity. In addition to this primary interaction, these compounds should have hydrophobic interactions with the protein which will promote binding affinity.

We were thus interested in site-specific cleavage of trypsin by these amidine-containing Schiff base metal chelates. The binding of the metal chelate to trypsin is expected to be a facile process due to electrostatic interaction by the amidinium group. In addition, this method may be applicable to other trypsin-like enzymes, such as plasmin, thrombin, and urokinase. In the present paper, Schiff base iron(III) chelates carrying an amidinium group (3a—i, 4a—i) have been prepared and the inhibitory activity of these chelates toward bovine trypsin has been studied. In addition, site-specific cleavage reactions of bovine trypsin by Schiff base copper(II) or iron(III) chelates in the presence of hydrogen peroxide and sodium ascorbate have been examined.

The amidine-containing iron(III) chelates (3a—i) were obtained by adding iron(III) nitrate to an alcoholic solution of a stoichiometric amount of ω-amino acid, 4-formyl-3-hydroxybenzamidine hydrochloride and potassium hydroxide. The reaction mixture was stirred for 3 h at room temperature, and the resulting reddish brown crystalline powder was collected and recrystallized from methanol-acetone. Chelates prepared as above were characterized by infrared and UV absorption spectroscopy and also by elemental analysis. Synthesis of the chelates (4a—i) was carried out in a similar manner using 3-formyl-4-hydroxybenzamidine hydrochloride.

The absorption spectrum of 3a (prepared from 1-alanine, R=CH3) showed marked absorption maxima at 430 nm (ε=3490) and 475 nm (ε=2930). The IR spectrum of 3a showed a C=O stretching frequency at 1630 cm⁻¹. These results indicate that iron(III) coordinates to carboxyl oxygen, imine nitrogen, and the phenolic oxygen of the Schiff base ligand.10 Elemental analysis indicated a 1:2 ratio of metal to Schiff base ligand.

The inhibitory activity of the amidine-containing iron(III) chelates towards trypsin was determined according to the reported procedure.11 Determination of inhibition constants (Ki values, the dissociation constants of enzyme–inhibitor complexes formed from trypsin and inhibitors) was carried out following the method of Dixon.12 Concentrations of inhibitors used in the kinetic analysis were in the range of 10^{-5} to 10^{-6} M, corresponding to their Ki values. A control experiment was carried out with bis(N-salicylidenedanilino)iron(III) chelate (6) lacking an amidinium group. The trypsic activity was entirely unaffected by 6 in this concentration range. At a higher concentration of 6, competitive inhibition with a Ki value of 1.4×10^{-3} M was found which is reasonable for the binding of an aromatic compound to trypsin.13 All iron(III) chelates behaved as potent competitive inhibitors and the Ki values are listed in Table 1. All were stronger inhibitors than the parent salicylaldehydes. The Ki values indicate that the chelates are involved in a highly specific interaction.

No pronounced difference in the inhibitory activity in relation to the ω-substituent was seen. Chelates derived from 3-formyl-4-hydroxybenzamidine exhibit mostly stronger binding affinity than those derived from 4-formyl-3-hydroxybenzamidine, but the difference was not significant. The binding affinities of iron(III) chelates for bovine trypsin were comparable to those of the copper(II) chelates reported previously (Ki, 1.1×10^{-5} to 1.1×10^{-6} M).13

Cleavage of the trypsin backbone resulting from specific binding of the copper(II) or iron(III) chelates was next inves-
tigated. A control experiment was carried out with \( N\)-salicylideneanilinato(aqua)copper(II) chelate (5) and with iron(III) chelate 6 lacking an amminium group. Analysis of the cleavage reaction was performed by sodium dodecyl sulfate (SDS)–PAGE (polyacrylamide gel electrophoresis) as shown in Fig. 1. When trypsin was incubated in buffer alone, several bands were observed (lane 2). The major band at about 24 kDa was assigned to \( \beta\)-trypsin (single chain form). However, a variety of peptides corresponding to 15, 12, 9 kDa and so on were also observed. It has been reported that the bands at 12 and 9 kDa are due to \( \alpha\)-trypsin (a single peptide bond Lys\( _{131}\)–Ser\( _{132}\) is cleaved), and the other bands are due to self-digestion.\(^{[14]}\) Lane 3 was the same as lane 2, in which \( \text{H}_2\text{O}_2\) and ascorbate were present. In the presence of \( \text{H}_2\text{O}_2\) and ascorbate, the effect of chelates 1a and 2a is shown in lanes 5.
and 6, respectively. Both lanes are characterized by a peptide band at 20 kDa. The band intensity, as measured by silver staining, was 15—16% that of β-trepsin (Fig. 2). A band corresponding to 20 kDa was originally seen in lanes 2 and 3, but its intensity was 4—5% that of β-trepsin (Fig. 2). This minor peptide band was likewise seen (4.3% of β-trepsin) in lane 4 where non-specific N-salicylidenealaninato(aqua)copper(II) chelate (5) was used instead of 1a or 2a (Fig. 2). Thus, the major peptide fragment (20 kDa) was due to site-specific cleavage of trepsin by the copper(II) chelates (1a, 2a). Cleavage by 1a or 2a was completely inhibited by addition of 0.05% SDS (data, not shown). Cleavage of trepsin by iron(III) chelates (3a, 4a) was also effective (lanes 8 and 9), though cleavage 6 was ineffective (lane 7), as shown in Fig. 2.

The cleavage site was estimated by using a trepsin model constructed from the atom coordinate data. The three-dimensional structure of trepsin suggested that the binding cavity of the active site was composed from three peptide chains, as shown in Fig. 3. In model-fitting, the amide nitrogen of the copper(II) chelate was oriented to the carbonyl carbon of the Asp residue at the bottom of the trepsin binding cavity, and the distance between the two atoms was kept at 2.9 Å. Cleavage by the copper(II) chelate may then be expected to occur at a position within the three peptide chains. The location of these peptide chains in the covalent structure of bovine trepsin is shown in Fig. 3. The molecular size of the newly formed fragment in Fig. 1 is about 20 kDa. Thus, cleavage may occur at chain C (between Gly and Ala). It is assumed that selective cleavage within the C chain results in the removal of a C-terminal peptide of about 3 kDa (Fig. 3, II). Thus, the peptide band observed at 20 kDa could be the remainder of the β-trepsin molecule.

Several studies have reported site-specific cleavage of proteins by designed affinity reagents. Two types of reagents have been described. The first is a reagent which exhibits non-covalent binding affinity to protein, and the second is a material that forms a covalent protein-protein conjugate prior to the cleavage reaction. In both cases, cleavage of the peptide bond occurs at the region where the metal attained close contact in its three-dimensional structure.

It was noticed that sharp cleavage bands on SDS–PAGE were obtained when metal chelates of the covalent conjugate type were used. In these cases, mild reaction conditions were applied. Reactions with non-covalent affinity reagents, in contrast, require drastic condition. They are usually accompanied by poorly resolved SDS–PAGE bands. An exception is the avidin-biotin system (Kₐ is as small as ca. 10⁻⁵ M).
During the course of the present work, cleavage under mild conditions was attempted but the result was essentially the same as the pattern in Fig. 1. Therefore the condition affording the best 20 kDa band was adopted.

The mechanism of the cleavage reaction with a EDTA–Fe complex has been studied using a model peptide system, and indicated that cleavage of polypeptide results from diffusible hydroxyl radicals, as well as peroxy species. In our case, hydroxyl radicals or active peroxy may also be responsible for the cleavage reaction.

The non-covalent affinity reagents described in this work have several advantages. They are easily prepared, and spontaneously bind to proteins. Extension of this work to a comparative study of the trypsin family is of interest from the clinical point of view. Determination of the precise cleavage site and analysis of other cleavage reagents is in progress.

Experimental

Materials  Bovine trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL). As a molecular weight marker, Protein Test Mixture 5 (Boehringer Ingelheim Bioproducts Partnership) was used.

Instruments  Melting points were determined on a Yanaco MP-500D. IR spectra were recorded with a JASCO FT/IR VALOR-III spectrometer. Absorption spectra were recorded with a U-2000 spectrophotometer (Hitachi).

Synthesis of p-Aminoisalicilidene-c-alaninato(aqua)copper(II) Hemihydrate (1a)  This compound was synthesized according to the previously reported procedure. Obtained in 65% yield as a dark green powder. mp 212–215 °C (dec.). IR (KBr): 1640 (C= N) cm⁻¹. UV λmax nm (ε): 374 (5610).

Synthesis of m-Aminoisalicilidene-c-alaninato(aqua)copper(II) (2a)  Synthesis was carried out as for 1a. Yield 62%, mp 230 °C. IR (KBBr): 1640 (C= N) cm⁻¹. UV λmax nm (ε): 374 (5270).

Synthesis of Potassium Bis(p-aminoisalicilidene-c-alaninato)iron(III) Trihydrate (3a)  Synthesis was carried out following the procedure reported for potassium bis(N-salicylidenedialaninato)iron(III). A mixture of 4-formyl-3-hydroxybenzamide hydrochloride (0.41 g, 2.04 mmol), l-alanine (0.15 g, 2.02 mmol), and potassium hydroxide (0.23 g, 4.10 mmol) in water–ethanol (1:1) (15 ml) was stirred for several minutes at room temperature. An ethanolic solution of iron(III) nitrate enneahydrate (0.41 g, 1.02 mmol) was then added. The solution was stirred for 3 h at room temperature and concentrated to dryness in vacuo. Recrystallization from methanol–acetone gave a reddish brown powder in 70.2% yield, mp 207–208 °C (dec.). IR (KBBr): 1630 (C= N) cm⁻¹. UV λmax nm (ε): 430 (3400), 475 (2860). Anal. Calcd for C₁₂H₁₀Cl₂FeKN₁₀O₁₁C: C, 38.39; H, 4.39; Cl, 10.30; N, 12.21. Found: C, 38.45; H, 4.68; Cl, 10.06; N, 12.49.

Synthesis of Potassium Bis(m-aminoisalicilidene-c-alaninato)iron(III) Trihydrate (4a)  Synthesis was carried out in the same manner as described above, using 3-formyl-4-hydroxybenzamide hydrochloride. Yield 56.7%, mp 202–203 °C (dec.). IR (KBBr): 1620 (C= N) cm⁻¹. UV λmax nm (ε): 450 (3790). Anal. Calcd for C₁₄H₁₀Cl₂FeKN₁₀O₁₁C: C, 38.39; H, 4.39; Cl, 10.30; N, 12.21. Found: C, 38.38; H, 4.68; Cl, 10.08; N, 12.45.

Determination of Inhibitory Activity (Kₗ) of Ion(III) Complexes  Enzyme concentration was determined by active site titration with p-nitrophenyl p'-guanidonobenzoate. Enzyme activity was determined by active site titration with p-nitrophenyl p'-guanidonobenzoate.