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 α-amino acid, metal ion, and salicylaldehyde. These chelates behaved as specific inhibitors of trypsin, with K_i values in the range 10^{-5}—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, H_2O_2, and ascorbate. Examination of the three-dimensional structure of trypsin suggests that cleavage occurred at a peptide bond within the Gly_{195}–Ala_{204} 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 bromide[7] 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 α-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 Asp_{177} residue[9] 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 α-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 L-alanine, R=CH_3) showed marked absorption maxima at 430 nm (ε=3490) and 475 nm (ε=2930). The IR spectrum of **3a** showed a C=N 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 (K_i 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}—10^{-6} M, corresponding to their K_i values. A control experiment was carried out with bis(*N*-salicylidenealaninato)iron(III) chelate (**6**) lacking an amidinium group. The tryptic activity was entirely unaffected by **6** in this concentration range. At a higher concentration of **6**, competitive inhibition with a K_i 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 K_i values are listed in Table 1. All were stronger inhibitors than the parent salicylaldehydes. The K_i values indicate that the chelates are involved in a highly specific interaction.

No pronounced difference in the inhibitory activity in relation to the α-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 (K_i, 1.1×10^{-5}—1.1×10^{-6} M).[8]

Cleavage of the trypsin backbone resulting from specific binding of the copper(II) or iron(III) chelates was next inves-

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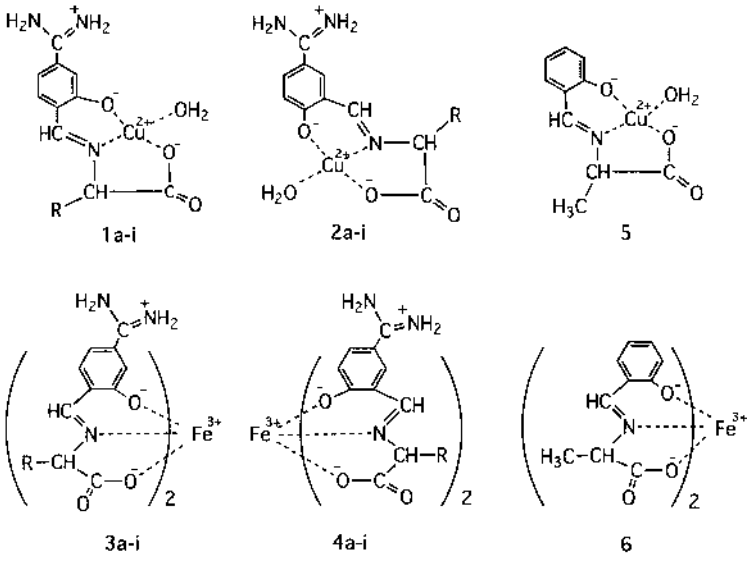


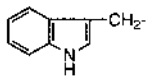
	Amino acid	R
a	L-Ala	CH_3-
b	L-Val	$(CH_3)_2CH$-
c	L-Leu	$(CH_3)_2CHCH_2$-
d	L-Ile	$CH_3CH_2CH(CH_3)$-
e	L-Phe	
f	L-Trp	
g	L-Met	$CH_3SCH_2CH_2$-
h	L-Ser	$CH_2(OH)$-
i	L-Thr	$CH_3CH(OH)$-

Chart 1

Table 1. Inhibition Constants of a Series of Iron(III) Chelates Derived from 4-Formyl-3-hydroxybenzamidine or 3-Formyl-4-hydroxybenzamidine for Trypsin-Catalyzed Hydrolysis of Benzoyl-L-arginine *p*-Nitroanilide at pH 8.0

Inhibitor	K_i (M)	Inhibitor	K_i (M)
Copper(II) chelates[a]			
1a	4.2×10^{-6}	**2a**	2.8×10^{-6}
Iron(III) chelates			
3a	1.6×10^{-6}	**4a**	1.8×10^{-6}
3b	9.3×10^{-6}	**4b**	2.0×10^{-6}
3c	2.9×10^{-5}	**4c**	4.2×10^{-6}
3d	1.2×10^{-5}	**4d**	5.9×10^{-6}
3e	7.7×10^{-6}	**4e**	4.2×10^{-6}
3f	3.1×10^{-6}	**4f**	1.8×10^{-6}
3g	3.9×10^{-6}	**4g**	1.9×10^{-6}
3h	6.3×10^{-6}	**4h**	7.6×10^{-6}
3i	5.8×10^{-6}	**4i**	3.6×10^{-6}
4-Formyl-3-hydroxy-benzamidine	2.8×10^{-5}	3-Formyl-4-hydroxy-benzamidine	1.2×10^{-5}

a) See reference 8.

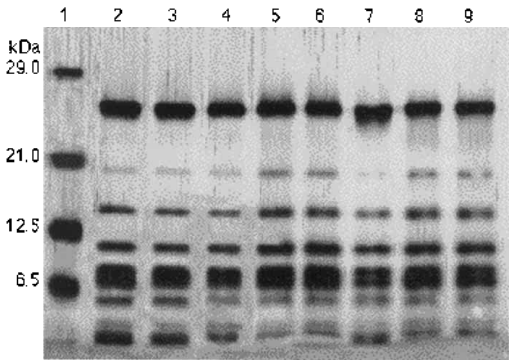


Fig. 1. SDS–PAGE Analysis of Incubates of Trypsin under Various Conditions

Lane 1, molecular weight marker; lanes 2—9, trypsin was incubated at 25 °C for 30 min with the following additions: lane 2, none; lane 3, H_2O_2+ascorbate; lane 4, **5**+H_2O_2+ascorbate; lane 5, **1a**+H_2O_2+ascorbate; lane 6, **2a**+H_2O_2+ascorbate; lane 7, **6**+H_2O_2+ascorbate; lane 8, **3a**+H_2O_2+ascorbate; lane 9, **4a**+H_2O_2+ascorbate.

tigated. A control experiment was carried out with *N*-salicylidenealaninato(aqua)copper(II) chelate (**5**) and with iron(III) chelate **6** lacking an amidinium 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 β-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 α-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 H_2O_2 and ascorbate were present. In the presence of H_2O_2 and ascorbate, the effect of chelates **1a** and **2a** is shown in lanes 5

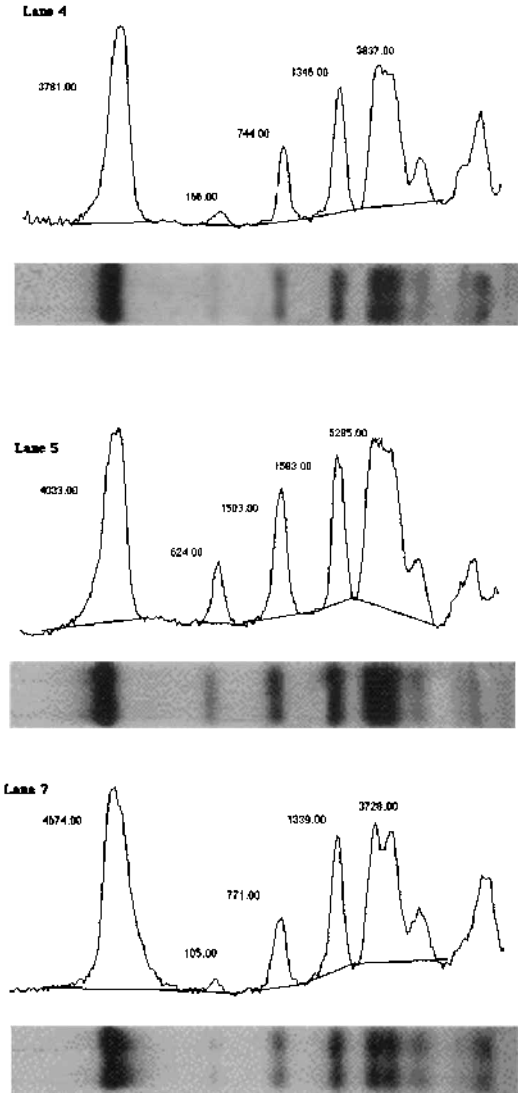


Fig. 2. Analysis of SDS–PAGE Pattern by Gel Scanner and Image Processing Computer Program (NIH Image)

Density ratios (20 kDa/β-trypsin) are 4.3, 15.5 and 2.2% for lanes 4, 5 and 7, respectively.

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-trypsin (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-trypsin (Fig. 2). This minor peptide band was likewise seen (4.3% of β-trypsin) 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 trypsin 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 trypsin by iron(III) chelates (**3a**, **4a**) was also effective (lanes 8 and 9), though chelate **6** was ineffective (lane 7), as shown in Fig. 2.

The cleavage site was estimated by using a trypsin molecular model constructed from the atom coordinate data.[15,16] The three-dimensional structure of trypsin suggested that the binding cavity of the active site was composed from three peptide chains, as shown in Fig. 3, I. In model-fitting, the amidine nitrogen of the copper(II) chelate was oriented to the

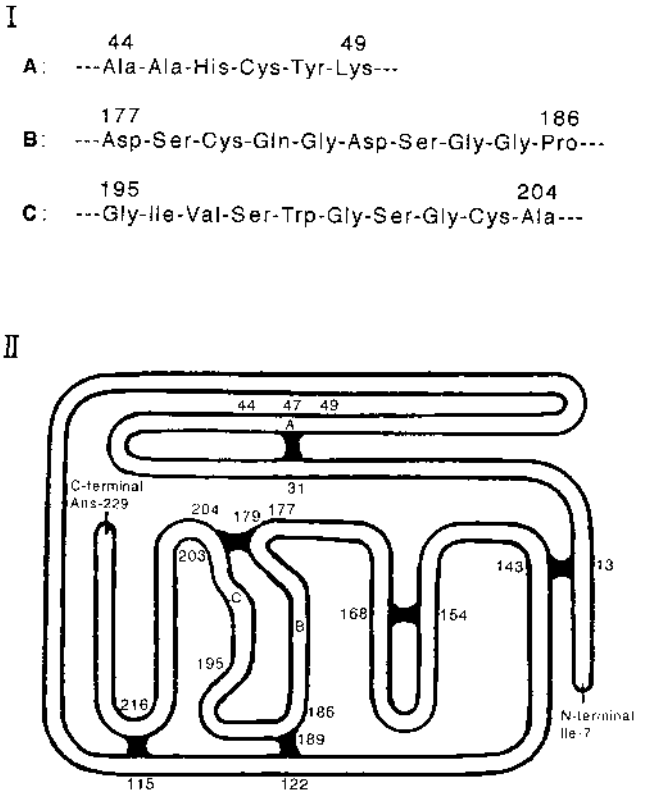


Fig. 3. The Binding Cavity of Trypsin Active Site Deduced by X-Ray Diffraction Data

The cavity was constituted by three peptide chains A, B and C (I). Each chain corresponds to the region A, B, and C of the covalent structure of the enzyme (II). The residue numbering is that for bovine trypsinogen.

carbonyl carbon of the Asp_{177} residue at the bottom of the trypsin binding cavity, and the distance between the two atoms was kept at 2.9 Å.[8,16] 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 trypsin is shown in Fig. 3, II.[17] The molecular size of the newly formed fragment in Fig. 1 is about 20 kDa. Thus, cleavage may occur at chain C (between Gly_{195}–Ala_{204}). 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-trypsin 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-reagent 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.[1—3] In these cases, mild reaction conditions were applied. Reactions with non-covalent affinity reagents, in contrast, require drastic condition.[4,6] They are usually accompanied by poorly resolved SDS–PAGE bands.[4] An exception is the avidin-biotin system (K_d is as small as *ca.* 10^{-15} M).[5]

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,[5,18] as well as peroxo species.[3] In our case, hydroxyl radicals or active peroxo 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*-Amidinosalicylidene-L-alaninato(aqua)copper(II) Hemihydrate (1a) This compound was synthesized according to the previously reported procedure.[8] Obtained in 65% yield as a dark green powder. mp 212—215 °C (dec.). IR (KBr): 1640 (C=N) cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 374 (5610).

Synthesis of *m*-Amidinosalicylidene-L-alaninato(aqua)copper(II) (2a) Synthesis was carried out as for **1a**. Yield 62%, mp 230 °C. IR (KBr): 1640 (C=N) cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 374 (5270).

Synthesis of Potassium Bis(*p*-amidinosalicylidene-L-alaninato)iron-(III) Trihydrate (3a) Synthesis was carried out following the procedure reported for potassium bis(*N*-salicylidenealaninato)iron(III).[10] A mixture of 4-formyl-3-hydroxybenzamidine hydrochloride[8] (0.41 g, 2.04 mmol), L-alanine (0.18 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 (KBr): 1630 (C=N) cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 430 (3400), 475 (2860). *Anal*. Calcd for $C_{22}H_{30}Cl_2FeKN_6O_9$: 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*-amidinosalicylidene-L-alaninato)iron-(III) Trihydrate (4a) Synthesis was carried out in the same manner as described above, using 3-formyl-4-hydroxybenzamidine hydrochloride.[8] Yield 56.7%, mp 202—203 °C (dec.). IR (KBr): 1620 (C=N) cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 450 (3790). *Anal*. Calcd for $C_{22}H_{30}Cl_2FeKN_6O_9$: 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 (K_i) of Iron(III) Complexes Enzyme concentration was determined by active site titration with *p*-nitrophenyl *p'*-guanidinobenzoate.[19] Enzyme activity was determined in 50 mM Tris–HCl buffer (containing 20 mM $CaCl_2$, pH 8.0) using benzoyl-L-arginine *p*-nitroanilide as a substrate. Hydrolytic rates in the presence of chelate were determined and the reciprocals of the rates were plotted as a function of chelate concentration following the method of Dixon.[12]

Cleavage Reaction Cleavage reaction was performed in a total volume of 250 μl of 50 mM Tris–HCl buffer (containing 20 mM $CaCl_2$, pH 8.0). A trypsin solution (10 μM) was incubated in the presence of the chelate (2 mM) for 5 min at room temperature. To this solution, a 10-fold excess of sodium ascorbate and a 5-fold excess of hydrogen peroxide over metal ion were added. The mixture was incubated for 30 min at 25 °C, and the reaction was terminated by addition of 125 μl of Tris–HCl buffer, pH 6.8 containing SDS (10%), 2-mercaptoethanol (25%), glycerol (10%) and bromophenol blue (0.02%). The mixture was then heated for 5 min in a boiling water bath. The products were analyzed by SDS–PAGE with silver-staining. The optical density of the gel was measured by a flat-head scanning device and the image data were processed by a computer program "NIH Image" (http://rsb.info.nih.gov/nih-image/).

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