COMPLEX FORMATION OF GUANIDINATED BOVINE TRYPSIN INHIBITOR (KUNITZ) WITH TRYPSIN, CHYMOTRYPSIN AND TRYPSINOGEN AS STUDIED BY THE SPIN-LABEL TECHNIQUE

H. R. WENZEL, H. TSCHESCHE, E. VON GOLDAMMER[+] and U. NETZELMANN[+]

Universität Bielefeld, Fakultät für Chemie, Lehrstuhl Biochemie, Postfach 8640, 4800 Bielefeld 1 and [+]*Ruhr-Universität Bochum, Institut für Biophysik, Postfach 102148, 4630 Bochum 1, FRG*

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1. Introduction

Studying the interaction between protein proteinase inhibitors and their target enzymes by physicochemical methods has often been hampered by the lack of suitable intrinsic spectroscopic signals, which change upon complex formation.

One approach to circumvent this difficulty is to introduce an indirect signal by coupling an indicator reaction to the reaction of interest. Examples are monitoring the enzymatic activity of free enzyme [2] or following the displacement of proflavin from the enzyme when the inhibitor is bound [3]. With all these indirect measurements careful studies as to possible side reactions and influences on the equilibrium and kinetic properties of the enzyme/inhibitor system have to be included. Another approach is to covalently attach reporter groups to the reaction partners. The association of chymotrypsin and the bovine trypsin inhibitor (Kunitz) for example was studied using covalently bound fluorescent labels [4]. Employing these techniques always comprises to show that the reporter groups are tolerable perturbations of the system under investigation.

Covalently attached nitroxyl radicals or spin-labels have been frequently used to monitor events in biological systems on the molecular level [5]. This paper examines the potentials of the spin-label method as a new tool to study the interaction between proteinases and their protein inhibitors. The fully guanidinated bovine trypsin inhibitor (Kunitz), which could be specifically spin-labelled at the amino-terminus and its complexes with trypsin, chymotrypsin and trypsinogen were chosen as a first test system.

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2. Experimental

2.1. *Materials*

Bovine trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and trypsinogen were obtained from Merck (Darmstadt). 3-Maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl was purchased from Syva (Palo Alto CA). The bovine trypsin inhibitor (Kunitz), Trasylol®, was kindly provided by Bayer (Wuppertal).

2.2. *Enzymatic assays*

Enzyme activities were measured spectrophotometrically using *N*-benzoyl-L-arginine-4-nitroanilide as substrate for trypsin and *N*-(3-carboxypropionyl)-L-phenylalanine-4-nitroanilide as substrate for chymotrypsin [6].

2.3. *Guanidination*

Guanidination of the native inhibitor with *O*-methylisourea was carried out essentially as in [7]. Amino acid analysis indicated quantitative conversion of all 4 lysine residues to homoarginine residues.

2.4. *Spin-labelling*

Guanidinated inhibitor, 10.6 mg (1.6 μmol), and 3-maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl, 3.6 mg (15 μmol), in 0.5 ml 50 mM phosphate buffer (pH 7.4) were shaken at room temperature for 140 h. Insoluble material was separated by centrifugation and the supernatant passed through a column of

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Sephadex G-25 fine (1.4 × 100 cm) equilibrated with 10 mM ammonium acetate. The fractions containing protein were pooled, lyophilized, dissolved in water and lyophilized again. Amino acid analysis of the product showed 5.16 arginine residues compared to 6 of the native inhibitor.

2.5. *ESR measurements*

All ESR experiments were carried out on a Varian E-12 X-band spectrometer at ambient temperature. The instrument settings were: scan range 10 mT, modulation amplitude 0.2 mT, microwave power 50 mW, time constant 0.3 s, scan time 4 min. Sealed disposable micropipettes of 50 μl or 100 μl content (Brand, Wertheim) were used as sample cells. The buffer was 0.1 M triethanolamine–HCl (pH 7.0). ESR titrations were carried out at constant total concentration of spin-labelled inhibitor, varying the enzyme concentration. The ratio $\alpha = [\text{inhibitor}]_{\text{free}}/[\text{inhibitor}]_{\text{total}}$ for a given enzyme concentration was calculated from the amplitude A of the low field line: $\alpha = (A-A_\infty)/(A_0-A_\infty)$. A_0 and A_∞ are the amplitudes at zero enzyme concentration and at an excess enzyme concentration sufficient to bring about virtually complete complex formation. Rotational correlation times were estimated as in [8] assuming isotropic motion. Dissociation constants were determined as in [9] or [10].

3. Results

3.1. *Guanidination and spin-labelling*

The bovine trypsin inhibitor (Kunitz) was guanidinated with *O*-methylisourea [7]. Quantitative amino acid analysis revealed that the conversion of all 4 lysine residues, including the reactive site lysine-15, to homoarginine residues was complete. The α-amino group of arginine-1 remained unaffected and lent itself as the attachment point for the nitroxyl moiety. We chose 3-maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl as spin-label reagent, because maleimides are known to react rather specifically with amino groups provided that no sulfhydryl groups are present [11]. Quantitative evaluation of the ESR spectrum (double integration and comparison with a standard solution) showed the incorporation of 0.87 nitroxyl groups/inhibitor molecule, a value in agreement with the loss of 0.84 arginine residues found by amino acid analysis. These figures indicate an almost complete spin-labelling of the inhibitor exclusively at its amino terminus.

3.2. *Inhibitory activities*

The inhibition curves in fig.1 show that the bovine inhibitor and its guanidinated and spin-labelled derivatives exhibit virtually the same specific inhibitory activities when tested against trypsin or chymotrypsin. The 1:1 stoichiometries remain unaffected by the derivatisations. From fig.1b it can be concluded that the dissociation constants for the 3 chymotrypsin complexes are ~70 nM. Because of the extremely weak dissociation of the trypsin complexes evident from fig.1a an estimation of the corresponding constants is not possible.

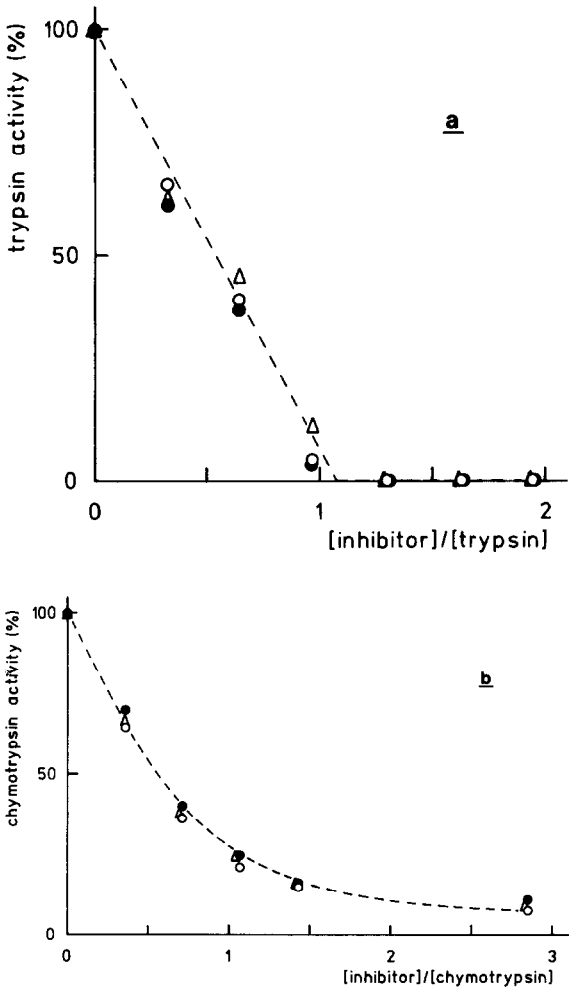

Fig.1. Inhibition of bovine trypsin (a) and bovine chymotrypsin (b) by the native (○), guanidinated (●) and the spin-labelled guanidinated (△) bovine trypsin inhibitor (Kunitz) (pH 7.8). Trypsin was 0.16 μM and chymotrypsin 0.8 μM; inhibitor concentrations varied.

3.3. *ESR spectra*

The spin-labelled guanidinated inhibitor in solution shows a 'weakly immobilized' ESR spectrum (fig.2a). Assuming isotropic motion, the rotational correlation time τ_c of the spin-label can be estimated to be ~2 ns [8], from which a particle diameter d of ~2.5 nm can be calculated, when the simple Debye model of rotation is used: $\tau_c = \pi\eta d^3/6\,kT$. Taking into account the geometry of the inhibitor, which is a pear-shaped molecule with a greatest length of 2.9 nm and a greatest diameter of 1.9 nm [12], this would seem to suggest that the nitroxyl group has only rather limited mobility relative to the protein.

When increasing amounts of trypsin, chymotrypsin or trypsinogen are added to the inhibitor solution, the ESR lines become broader and their amplitudes decrease. With complete complex formation a rotational correlation time of ~5 ns can be determined from the corresponding spectrum. These spectral changes are exemplified in fig.2 by a series of ESR spectra resulting from a chymotrypsin titration. The appearance of several isoclinic points suggests the existence of an equilibrium between 2 populations of spin-labels [13], one attached to free inhibitor and the second attached to the enzyme/inhibitor complex.

3.4. *Complexes with proteinases*

The fraction of free inhibitor at each point of a titration can be calculated from the ESR amplitudes (see section 2). The low field line was chosen, because it experiences the greatest changes. Fig.3 shows the resulting ESR titration curves with trypsin and chymotrypsin. A 1:1 stoichiometry is evident for both proteinase/inhibitor complexes. The dissociation constant for the chymotrypsin/spin-labelled guanidinated inhibitor complex can be estimated from the non-linear part of the curve to be ~80 nM. This value is in the same order of magnitude as the dissociation constant of 11 nM determined by kinetic measure-

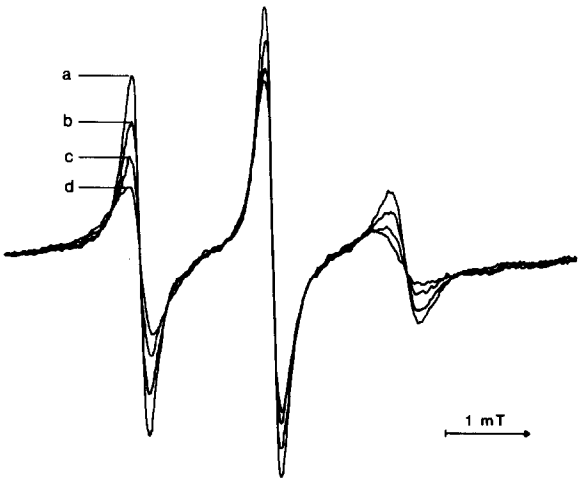

Fig.2. ESR spectra showing a titration of spin-labelled guanidinated inhibitor with bovine chymotrypsin (pH 7.0). Inhibitor was 50 μM. Chymotrypsin concentrations: (a) 0 μM; (b) 20 μM; (c) 40 μM; (d) 60 μM.

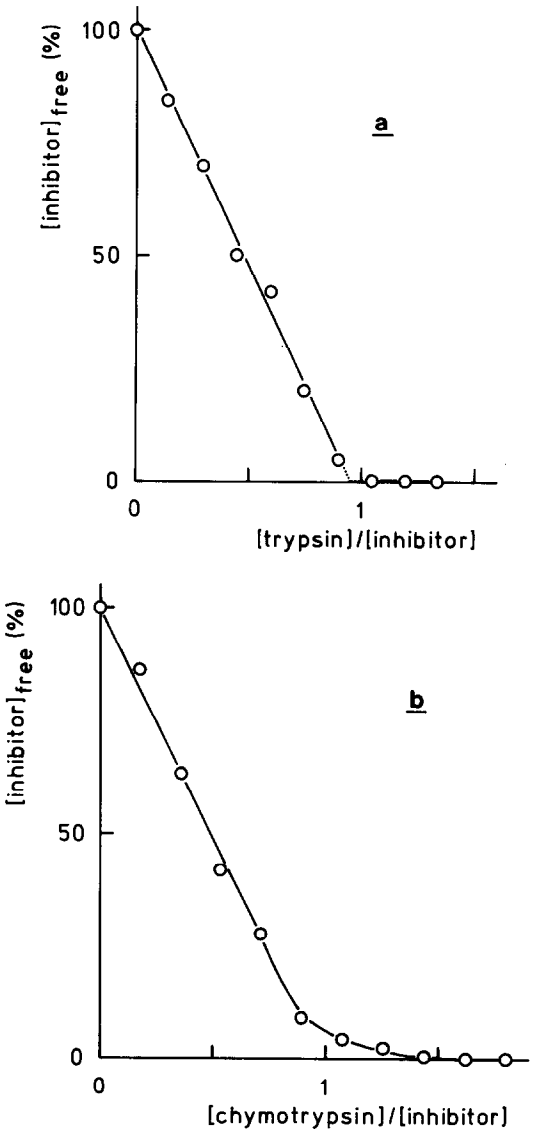

Fig.3. ESR titration curves of spin-labelled guanidinated inhibitor with bovine trypsin (a) and bovine chymotrypsin (b) (pH 7.0). Inhibitor was 50 μM; enzyme concentrations varied.

ments at pH 8.0 for the complex of chymotrypsin and Kunitz inhibitor specifically guanidinated at lysine-15 [14].

3.5. *Complexes with trypsinogen*

Trypsinogen is known to form a relatively weak 1:1 complex with the bovine trypsin inhibitor (K_d 2 μM at pH 8.0) [15,16]. ESR titration of spin-labelled guanidinated inhibitor with trypsinogen also indicates a 1:1 complex (K_d 20 μM at pH 7.0). The binding can be significantly enhanced by addition of the dipeptide Ile–Val. The reduction of the ESR amplitude at 50 μM trypsinogen and 50 μM inhibitor concentration upon addition of 5 mM Ile–Val corresponds to a decrease of the dissociation constant to ~7 μM.

The dipeptides Met–Val, Leu–Val, Ala–Val and Ala–Ala are without any appreciable effect. Thus, proenzyme, inhibitor and Ile–Val specifically form a ternary complex.

This result corroborates the detailed studies on the mechanism of trypsinogen activation [16,17]. The activation is brought about by the enzymatic removal of the N-terminal hexapeptide followed by the binding of the newly formed α-amino group of Ile-16 to the carboxylate side chain of Asp-194. This interaction is of crucial importance for the stability and function of the active trypsin. The dipeptide Ile–Val can play the structural role of the N-terminus of the active enzyme and introduce a transition of trypsinogen to a trypsin-like state. This reveals itself in the enhanced binding of the spin-labelled guanidinated inhibitor.

4. Discussion

This paper introduces spin-labelling in combination with ESR spectroscopy as a new and promising tool to study the interaction between proteinases and their protein inhibitors. A maleimide spin-label was rather rigidly attached to the terminal amino-group of the guanidinated bovine trypsin inhibitor (Kunitz), where it did not appreciably change the specific inhibitory activity. The paramagnetic nitroxyl moiety provided ESR signals, which allowed for direct quantitative monitoring of the complex formation between inhibitor and trypsin, chymotrypsin or trypsinogen. A 1:1 stoichiometry was found in all cases, the dissociation constants were somewhat increased as compared with the unlabelled systems.

The high sensitivity of the ESR technique makes it possible to detect reasonable signals even in ~10 μl of a μM spin-label solution. The signal-to-noise ratio can certainly be improved by the use of spectral accumulation and other recent technical advances. Other attractive aspects of the spin-label method are the possibility to measure in optically opaque solutions and, usually, the absence of interfering signals from the environment.

The inherent disadvantage of spin-labelling, which it has in common with any reporter group technique, is the perturbation of the system by the addition of the reporter molecule. However, with the broad selection of spin-labels now available with specificities for distinct amino acid side chains it should be feasible to minimize this problem. Thus it can be anticipated that our spin-label approach may be an additional useful tool also for other studies of protein/protein association.

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