Spin-label studies on protein proteinase inhibitors: Complex formation and conformational changes of the bovine trypsin inhibitor (Kunitz)

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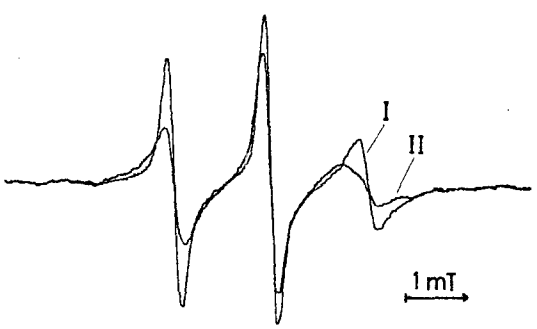
Inhibition of serine proteinases by the bovine inhibitor (Kunitz) results from complex formation between enzyme and inhibitor. In order to study these protein-protein interactions by electron spin resonance we decided to spin-label the inhibitor. Three different approaches were exploited to specifically attach a nitroxyl moiety at the N-terminal amino group which is known to be remote from the reactive site region.

a) Transamination with glyoxylate and reaction of the newly formed carbonyl function with the hydrazone of 2,2,6,6-tetramethyl-4-piperidone-1-oxyl.
b) Guanidination of the four lysine ε-amino groups and coupling of 4-isothiocyanato-2,2,6,6-tetramethylpiperidine-1-oxyl to the α-amino group.
c) Guanidination and addition of 3-maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl to the α-amino group.

The third reaction sequence proved to be the method of choice because the label was bound with high yield and experienced only limited rotational mobility relative to the protein. Bovine inhibitor (Kunitz) labelled according to method c) was therefore used for the following experiments.



The rotational correlation time of 2 ns estimated for the spin-labelled inhibitor (50 μM in 0.1 M triethanolamine buffer, pH 7.0, 293 K, spectrum I) increased to 5 ns when an equimolar amount or excess of bovine trypsin was added (spectrum II). These values correspond to an increase of the molecular weight when the inhibitor (MW 7000) combines with trypsin (MW 24000) to yield the complex (MW 31000). It is obvious that the spectral changes can be used to establish titration curves when the [trypsin]/[inhibitor] ratio is changed. Complex formation could also be demonstrated with porcine pancreatic kallikrein and bovine chymotrypsin. Binding of the inhibitor to bovine trypsinogen was relatively weak but could be increased by addition of the dipeptide Ile-Val. This indicates formation of a ternary complex.

ESR spectroscopy was also used to monitore conformational changes of the spin-labelled inhibitor induced by temperature changes. Arrhenius plots of the rotational correlation time revealed two break points in the temperature range from 20°C to 95°C. A reversible transition occurred at 44°C and a second one which was irreversible at 75°C (inhibitor concentration 100 μM in 0.1 M phosphate buffer, pH 7.0).

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