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^{13}C NMR STUDIES OF THE BINDING OF SOYBEAN TRYPSIN INHIBITOR TO TRYPSIN

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SUMMARY: NMR studies of the complex between trypsin and soybean trypsin inhibitor with 1-^{13}C-arginine and modified inhibitor with 1-^{13}C-lysine show that these complexes involve almost exclusively non-covalent binding of the inhibitor to the enzyme for trypsin/^{13}C-Lys-inhibitor at pH 6.5 and 8.1 and for trypsin/^{13}C-Arg-inhibitor at pH 5.0. At pH 7.1 for trypsin/^{13}C-Arg-inhibitor both non-covalent and acyl enzyme forms are observed. Under no conditions did we observe evidence for a tetrahedral adduct between enzyme and inhibitor.

INTRODUCTION

Soybean trypsin inhibitor (Kunitz) (STI) is one of many proteinase inhibitors found in a variety of animal and plant tissues (1). STI has a molecular weight of 22,000 and binds to trypsin with an association constant (K_a) of 10^9 M^{-1} at pH 8.0; K_a decreases rapidly with decreasing pH. STI has a reactive amide bond between arg 63 and ile 64 which can be cleaved by trypsin under appropriate conditions (2). These and many other observations support the view that naturally occurring trypsin inhibitors function as substrate analogues which, however, form much more stable complexes with the enzyme than do normal substrates (3).

A central question in the molecular function of these inhibitors has been the nature of the interactions between the residues at the catalytic site of the enzyme and the peptide bond between arg 63 and ile 64: does the complex involve simple Michaelis binding between enzyme and inhibitor (4); is the complex an analogue of the acyl-enzyme intermediate with an ester bond between the hydroxyl group of ser 195 and the carbonyl carbon of arg 63 (5), or does the complex exist as a tetrahedral adduct in which the hydroxyl group of ser

T/STI = trypsin/soybean trypsin inhibitor complex.
STI-Arg* = STI with arg 63 replaced by 1-^{13}C-Arg.
STI-Lys* = STI with arg 63 replaced by 1-^{13}C-Lys.

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195 of the enzyme has been added across the carbonyl group of the peptide bond (6,7)?

In this work we have used cmr to probe this question by observing the T/STI complex in which arg 63 of STI has been replaced either with 1-^{13}C-arg (T/STI-Arg*) or 1-^{13}C-lys (T/STI-Lys*) (8).

MATERIALS AND METHODS

Materials

Carbon-13 labeled KCN (90% enriched) was purchased from Merck, Sharp and Dohme. Soybean trypsin inhibitor was obtained from Gallard-Schlesinger Chemical Corp. Lyophilized bovine trypsin (lot TRL 34B884) was purchased from Worthington Biochemical Corp. CPase B was purified from the acetone powder extract of porcine pancrease (Pancreatin Grade II and III from Sigma), as described (9). 1-^{13}C-Arginine (10) and 1-^{13}C-lysine (11) were prepared by published procedures.

Preparation of the Complex Between Trypsin and 1-^{13}C-Arginine of 1-^{13}C-Lysine Soybean Trypsin Inhibitor. Enzymic replacement of the arg 63 residue in the reactive site of soybean trypsin inhibitor by 1-^{13}C-arginine or 1-^{13}C-lysine followed the published procedure (8).

Methods

Carbon-13 nmr spectra

A Varian XL-100-15 nmr spectrometer operating at 25.14 MHz in the FT mode was used to record the cmr spectra. In general, spectra were recorded with the following parameters: 6000 Hz sweep width, 0.2 sec acquisition time, and 90° pulse (at 60 μsec). Proton noise decoupling was used. Digital line broadening of 6 Hz, corresponding to a sensitivity enhancement of 0.05, was applied to the FID before Fourier transformation. Temperature of the probe was 31-32°C. Difference spectra were obtained by computer subtracting a cmr spectrum of unlabeled T/STI complex taken at pH 7.2 from each cmr spectrum of the labeled complexes. (The ^{13}C-natural abundance spectrum of the T/STI complex does not change observably in the range pH 3-8.) The difference spectra program was provided by Dr. S. H. Smallcombe of Varian Associates.

Sample preparation

Generally, solutions of T/STI complex (1-2mM) were made up in 0.2M KCl and transferred to a 12 mm nmr tube fitted with vortex plugs and a D_2O locking capillary. The pH's of the solutions were measured with a Radiometer Model PM 26 pH meter. Adjustment of pH was done with either 1N HCl or 1N NaOH.

RESULTS AND DISCUSSION

Figure 1 shows ^{13}C difference spectra for T/STI-Lys* obtained at pH 8.1, 6.5 and 3.0. Figure 2 shows difference spectra for T/STI-Arg* at 7.1 and 5.0. A formal difference spectrum of T/STI-Arg* at pH 3.0 was not obtained, but the

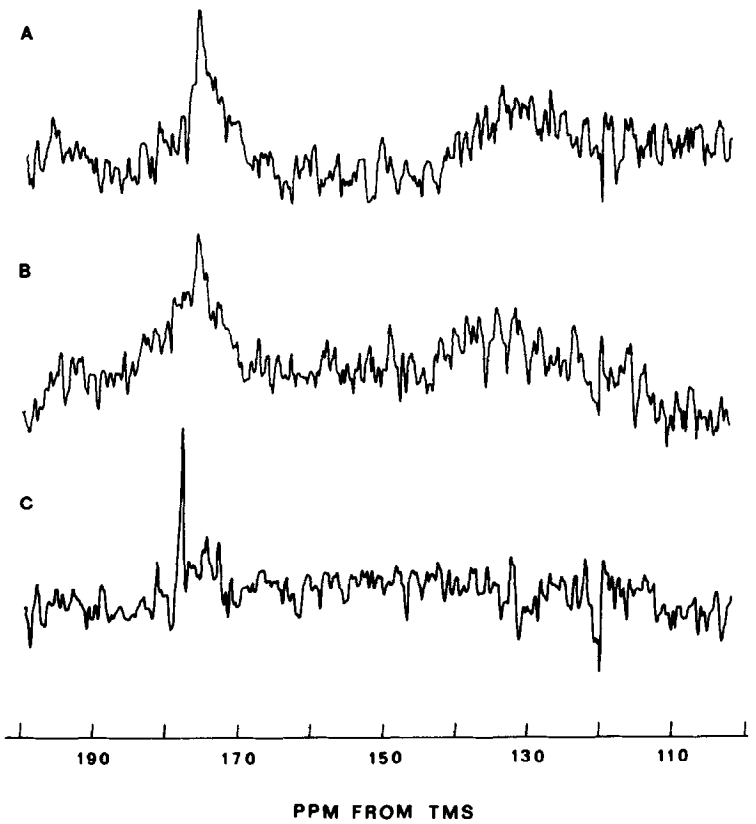

Figure 1. The cmr difference spectra of trypsin/soybean trypsin inhibitor complex (carbon-13 enriched at C-1 carbon of lys-64 of STI): spectrum taken at pH (A) 8.1; (b) 6.5; and (c) 3.0. Sweep width is 2500 Hz.

^{13}C spectrum of the complex shows a peak at 177.5 ppm which is absent in the natural abundance spectrum of unenriched T/STI. Table I lists the chemical shifts of the resonances observed for T/STI-Lys* and T/STI-Arg* as well as for C-1 of arginine and lysine near neutral pH.

A significant difference between the T/STI-Lys* and T/STI-Arg* is apparent near neutral pH where T/STI-Arg* shows two resolved ^{13}C resonances in contrast to the single resonance seen for T/STI-Lys*. At acidic pH both T/STI-Lys* and T/STI-Arg* show only a single resonance..

The positions of the ^{13}C resonances for the complex eliminate the presence of significant amounts of tetrahedral adduct. Such a tetrahedral species should have a ^{13}C chemical shift near that for C-1 of a substance such as

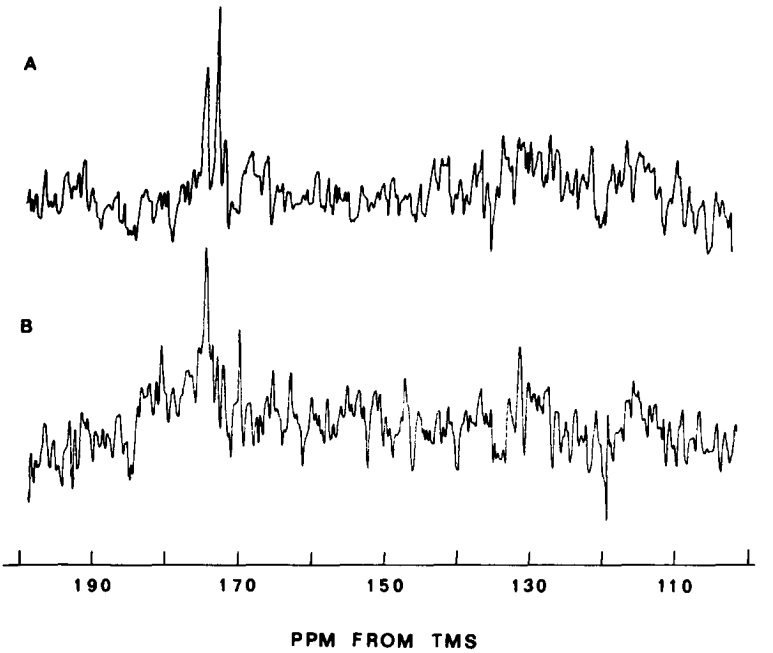

Figure 2. The cmr difference spectra of trypsin/soybean trypsin inhibitor complex (carbon-13 enriched at C-1 carbon of arg-64 of STI): spectrum taken at pH (A) 7.1 and (B) 5.0. Spectrum width is 2500 Hz.

Table I: Carbon-13 Chemical Shifts for the C-1 Carbon of Arginine and Lysine, Free and in Active Site of STI.

Arginine		Lysine	
pH	ppm	pH	ppm
6.0	175.5	6.8	175.7
T/STI-Arg*		T/STI-Lys*	
pH	ppm	pH	ppm
7.1	173.3, 174.9†	8.1	175.2
5.0	174.8	6.5	175.2
2.9	177.5	3.0	177.6

†A single resonance for T/STI-Arg* at 174.7 ppm has been observed independently (D. E. Neves, J. L. Markley, M. E. Welch and M. Laskowski, Abstract submitted for 1979 FASEB). We thank Professor Markley for informing us of these results prior to their publication.

ethyl orthoformate which falls at 127.2 ppm (12) or even, possibly, dimethoxymethane at 94.2 ppm (12). These positions are far removed from the region of 170-180 ppm which is characteristic for trigonal carbonyl carbons in ester, amide, or carboxyl groups (13).

Three possibilities remain for the bonding present in the T/STI complexes: (i) intact amide bond in a native inhibitor which is held to the trypsin in a non-covalent Michaelis complex; (ii) an acyl enzyme species and (iii) a non-covalent, Michaelis complex between trypsin and a modified inhibitor in which the 64-65 bond has been hydrolyzed; C-1 of arginine (or lysine) would then exist as a carboxylic acid or carboxylate anion.

We identify the resonances observed at 177.5 ppm for T/STI-Arg* at pH 2.9 and at 177.6 for T/STI-Lys* at pH 3 as arising from C-1 of carboxylic acids probably in equilibrium with small amounts of the corresponding anions. The pH dependent changes we observe for the complexes between neutral pH and pH 3 (a downfield shift of 2.0 ppm for T/STI-Arg* and of 1.9 for T/STI-Lys*) accord with cmr studies of many peptides (14,15) and confirm previous evidence that the complex dissociates at low pH at which pH the inhibitor exists largely in a modified form in which the arg 63-ile 64 bond has been hydrolyzed.

Near pH 7, the T/STI-Arg* complex shows two resonances at 173.3 ppm and 174.9 ppm, which fall in the range appropriate for an amide and ester group suggesting that, at this pH, T/STI-Arg* exists as a mixture of (i) a non-covalent complex between enzyme and inhibitor with an intact amide bond between arg 63 and ile 64 and (ii) an acyl enzyme form in which an ester exists between ser 195 of trypsin and arg 63 of inhibitor. These two forms are in slow exchange on the nmr time scale. Although chemical shifts of carbonyl carbons of esters and amides fall in the same range, those of amides generally occur at somewhat lower field than those of the corresponding esters. On this basis, we tentatively assign the signal at 174.9 ppm to the non-covalent complex between native inhibitor and enzyme and the signal at 173.3 ppm to the acyl enzyme complex. The presence of both non-covalent and acyl enzyme complexes for T/STI-Arg* may also account for the X-ray diffraction results (6,7) in which the observed electron density might be interpreted as being consistent with a tetrahedral adduct. Possible ambiguities in interpretation of these maps have been recognized (7b) and X-ray studies of the complex

between anhydrotrypsin and bovine trypsin inhibitor (6b) suggest that the carbonyl carbon of Lys 15 is distorted pyramidally but that the distance between it and ser 195 O^{γ} is too great for significant covalent bond formation. (Another formal possibility is that the two signals reflect two distinct conformations of T/STI-Arg* which interconvert slowly on the nmr time scale.)

Comparison between C-1 of free arginine (175.5 ppm) and T/STI-Arg* with intact inhibitor (174.9) shows an upfield shift of 0.6 ppm on incorporation of free arginine as an internal residue in the inhibitor. Incorporation of lysine into the T/STI-Lys* complex shows an upfield shift of 0.5 ppm (from 175.7 ppm in free lysine to 175.2 in the complex). These values agree with upfield shifts of 1.5±1.0 ppm (14) and 0.8 to 1.9 ppm (15) observed for C-1 of amino acids upon incorporation into internal positions in peptides.

At pH 5 in both T/STI-Lys* and T/STI-Arg* we observed only a single resonance for the complex at a position assigned to the intact inhibitor in a non-covalent Michaelis complex with trypsin. A recent study found $\sim$ 10% of the acyl enzyme form present in T/STI at pH 5.2 (16). This is consistent with our observation of the acyl enzyme form at neutral pH and its apparent absence in the cmr spectrum at pH 5.0 as 10% acyl enzyme is just below our observational limits.

We observe only one resonance, characteristic of an amide carbonyl, for T/STI-Lys* indicating that this complex exists almost exclusively as non-covalently bound amide over the pH range 5-8. Thus, the substitution of lysine for arginine modifies the molecular details of the interaction between inhibitor and enzyme even though this substitution causes little perturbation in the thermodynamics of binding (8).

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