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Soybean Trypsin Inhibitor (Kunitz) and Its Complex with Trypsin. Carbon-13 Nuclear Magnetic Resonance Studies of the Reactive Site Arginine[†]

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ABSTRACT: The reactive site Arg^{63}–Ile^{64} peptide bond of soybean trypsin inhibitor (STI) is preferentially and reversibly hydrolyzed by trypsin. A modification of the method of Sealock & Laskowski [Sealock, R. W., & Laskowski, M., Jr. (1969) *Biochemistry 8*, 3703–3710] was used to replace Arg^{63} of STI with [^{13}C$^{\zeta}$]arginine and [^{13}C$^{\circ}$]arginine. Protein purity was established by disc gel electrophoresis. The ^{13}C NMR spectrum of [[^{13}C$^{\zeta}$]Arg^{63}]STI revealed a single peak with chemical shift 157.81 ppm [downfield from $(CH_3)_4Si$] above the background spectrum of unenriched STI. When sufficient bovine or porcine trypsin was added to complex all but a small fraction of the [[^{13}C$^{\zeta}$]Arg^{63}]STI, the peak assigned to Arg^{63} C$^{\zeta}$ shifted to 158.04 ppm. We conclude from the negligible chemical shift change that there is no significant proton transfer from the guanidinum group upon complex formation. The ^{13}C NMR spectrum of [[^{13}C$^{\circ}$]Arg^{63}]STI revealed one peak at 173.8 ppm. In labeled modified STI, [[^{13}C$^{\circ}$]-Arg^{63}]STI(63,64), the chemical shift of the labeled carbon was pH dependent (179.0 ppm at pH 7.0; 174.7 ppm at pH 2.9). When a half-stoichiometric amount of bovine trypsin was added to labeled STI, two ^{13}C NMR peaks were observed, at 173.8 and 174.7 ppm. Disc gel electrophoresis identified the two species as [[^{13}C$^{\circ}$]Arg^{63}]STI and the complex, [[^{13}C$^{\circ}$]-Arg^{63}]STI–trypsin. After treatment with sufficient bovine trypsin to yield only a small excess of labeled STI, a single peak remained at 174.7 ppm. The absence of a large upfield shift of the [^{13}C$^{\circ}$]Arg^{63} peak upon complexation establishes that the STI–trypsin complex is not a covalent, fully tetrahedral adduct. A covalent, fully tetrahedral intermediate structure has been proposed for the STI–trypsin complex [Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4212–4228]. Although a covalent, fully tetrahedral structure was proposed initially for the pancreatic trypsin inhibitor–trypsin complex [Rühlmann, A., Kukla, O., Schwager, P., Bartels, K., & Huber, R. (1973) *J. Mol. Biol. 77*, 417–436], subsequent X-ray experiments have ruled this out. The present ^{13}C NMR results do not allow us to distinguish between a complex in which C$^{\circ}$ is completely trigonal and the structure recently proposed for PTI–trypsin based on X-ray studies, where C$^{\circ}$ is partially tetrahedral due to constraints imposed by the enzyme–inhibitor contact [Huber, R., & Bode, W. (1978) *Proc. FEBS Meet. 47*, 15–34].

It is well-known that ^{13}C NMR chemical shifts cover a much broader range (200 ppm) than ^{1}H NMR shifts (10 ppm) and are highly sensitive to the electronic structure (bond order) of the carbon nucleus (Stothers, 1972). For example, the C_2-carbonyl of fructose has a chemical shift of 217.4 ppm in the aldehyde form and 107.5 ppm in the α-furanose form (Funcke & Klemer, 1976). In contrast, the environmental perturbations of ^{13}C NMR chemical shifts in proteins are relatively small (Allerhand, 1979). Apart from protonation shifts and ring current shifts, the origins of environmental shifts in proteins are largely obscure at present (Wüthrich, 1976; Allerhand, 1979). Selective isotopic replacement of NMR active nuclei provides the most successful means of simplifying NMR spectra of proteins and of making unambiguous peak assignments (Markley, 1972). Isotopic substitution in proteins has been achieved by biosynthesis (Markley et al., 1968; Browne et al., 1973) or by peptide synthesis (Chaiken et al., 1973) starting with isotopically labeled amino acids, and by selective isotopic exchange reactions in intact proteins (Meadows et al., 1968; Markley, 1975; Jones et al., 1975). Enzymatic semisynthesis (Sealock & Laskowski, 1969; Offord, 1969) offers an alternative approach having several attractive features: (1) the method can be used to label proteins from higher organisms for which biosynthetic techniques are difficult; (2) the method can be used to label a single residue rather than all residues of a given kind as is normally accomplished by biosynthesis; (3) the method requires a smaller amount of expensive isotopically labeled amino acid than biosynthesis, and much of this can be recovered. A disadvantage of enzymatic semisynthesis is its lack of generality; however, in some cases this can be overcome (Homandberg et al., 1978; Homandberg & Laskowski, 1979).

Sealock & Laskowski (1969) reported a procedure for the enzymatic replacement of Arg^{63} of soybean trypsin inhibitor (Kunitz) (STI)[1] with a lysine residue. We describe here an extension of this method whereby we have labeled Arg^{63} with 90% carbon-13 for ^{13}C NMR spectroscopy. We report NMR

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[1] Abbreviations used: STI, soybean trypsin inhibitor (Kunitz); PTI, bovine pancreatic trypsin inhibitor (Kunitz); STI–trypsin, the stable complex formed between STI and trypsin; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); primed amino acid numbers refer to trypsin identified by the residue number of the homologous amino acid in the chymotrypsinogen sequence; pH*, uncorrected pH meter readings of a $^{2}H_2O$ solution made with a glass electrode standardized in $^{1}H_2O$ buffers; AcArgOMe, N^{α}-acetyl-L-arginine ester. An internal peptide bond hydrolysis in a protein is indicated by a comma between the residue numbers (from the intact sequence) of the amino acids on either side: for example, STI(63,64) represents STI with the peptide bond between residues 63 and 64 hydrolyzed whereas des-Arg^{63}-STI(62,64) represents STI with residue 63 removed (Kowalski & Laskowski, 1976). The reactive site peptide bond of an inhibitor is identified as P_1–$P_{1'}$ according to the notation of Schechter and Berger (1967). The crystallographic numbering system is used to designate atoms within residues; in particular, C$^{\circ}$ refers to 1-C.

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results for two ^{13}C-labeled inhibitors and their complexes with trypsin. Unfortunately, our results cannot be compared with those from an independent ^{13}C NMR study of the STI–trypsin complex (Hunkapiller et al., 1979).[2] The following paper in this issue reports ^{13}C NMR studies on a ^{13}C-labeled bovine pancreatic trypsin inhibitor (PTI) analogue and its complexes with trypsin, trypsinogen, and anhydrotrypsin (Richarz et al., 1980). Their experiment is not quite analogous to ours. In their experiment, the semisynthetic procedure led to the hydrolysis of the Arg^{39}-Ala^{40} peptide bond and to subsequent removal of Arg^{39}. Richarz et al. (1979, 1980) argue that this additional modification has little effect on their results. We agree. However, in our experiment on STI there are no additional bond hydrolyses.

STI, one of the best characterized inhibitors of serine proteinases, has a molecular weight of 20 100 based on its amino acid sequence (Koide et al., 1972). It has a large trypsin association equilibrium constant of 10^{11} at pH 8.3 (Finkenstadt et al., 1974). Trypsin specifically hydrolyzes the reactive site peptide bond Arg^{63}–Ile^{64} of STI. The standard mechanism for this reaction, which is general for the major class of proteinase inhibitors (Laskowski et al., 1975; Quast et al., 1978), is

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^* \qquad (1)$$

where E is free enzyme, I is virgin inhibitor (reactive site peptide bond intact), I* is modified inhibitor (reactive site peptide bond hydrolyzed), and L and L* are loose, noncovalent complexes of enzyme and virgin or modified inhibitor, respectively. Species C is the stable inhibitor–enzyme complex; X is a precomplex species which has been observed in the association of modified bovine pancreatic trypsin inhibitor (Kunitz), PTI(15,16), with trypsin (Quast et al., 1978).

The characterization of proteinase–inhibitor complex (C in eq 1) has gone through several stages of development as X-ray diffraction data were refined and extended. At 2.8-Å resolution, Rühlmann et al. (1973) reported the PTI–bovine trypsin complex to be "a tetrahedral adduct with a covalent bond" between the active site $Ser^{195'}$ O^{γ} of trypsin and the reactive site Lys^{15} C° of PTI. Sweet et al. (1974) reported the analogous result for the STI–porcine trypsin complex at 2.6-Å resolution. However, in a further refinement of the PTI–bovine trypsin complex to 1.9-Å resolution, Huber et al. (1974, 1975) reported the distance between the $Ser^{195'}$ O^{γ} and Lys^{15} C° to be 2.6 Å. This distance is far too long for a normal covalent bond (1.43 Å), (Pauling, 1960) and is only slightly less than a van der Waals contact distance. In the refined structure, the C°-oxygen of Lys^{15} is bent out of the C^{α}–C°–N plane by −34°, halfway between a trigonal (0°) and tetrahedral (−54°) conformation (see Figure 1b). Further, only slight changes in the PTI complex were reported when trypsin was replaced by anhydrotrypsin (Huber et al., 1975), an inactive derivative with the active site $Ser^{195'}$ converted to a dehydroalanine residue. Thus, the distortion of the Lys^{15} C° is currently ascribed (Huber & Bode, 1978) to the interactions of the C°-oxygen with the oxyanion binding hole (Robertus et al., 1972) rather than to the approach of $Ser^{195'}$.

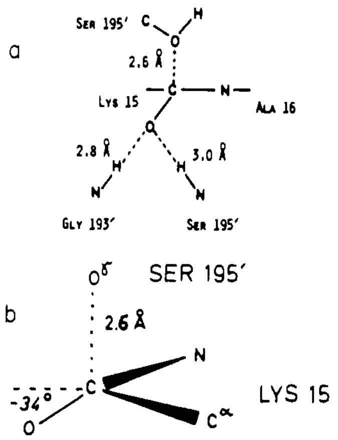

FIGURE 1: Schematic representations of the PTI–trypsin complex according to X-ray studies. (a) The catalytic residues and the scissile peptide (Huber & Bode, 1978); (b) $Ser^{195'}$ O^{γ} and the susceptible peptide group of PTI (Bode, 1979).

Selective ^{13}C labeling of the Arg^{63} C° permits one to determine its chemical shift in STI, STI(63,64), and STI–trypsin. The results establish unequivocally the absence of a covalent tetrahedral bond joining the two proteins in STI–trypsin (Sweet et al., 1974). Selective ^{13}C labeling of the guanido carbon of Arg^{63} in STI allows one to compare ^{13}C NMR parameters for a residue that is thought to be on the surface of the protein and free to interact with the polar solvent (in free STI) with those of the same residue when it is immobilized and buried in the largely hydrophobic specificity pocket of trypsin (in STI–trypsin).

Experimental Procedures

Materials. Clivorine was a generous gift from Professor F. Santavý. The sources of proteins were as follows: carboxypeptidase B, Worthington (COBC 54E521); soybean trypsin inhibitor, Sigma Chemical Co. (93C-8140); porcine trypsin, Novo (6241 DF); bovine trypsin, Worthington (TRL 37A870); acylase I, Sigma (67C-0211). $^{2}H_2O$ was purchased from Bio-Rad Laboratories and was purified by passage through a Chelex (Bio-Rad) column to remove paramagnetic impurities immediately prior to use. Sources of other materials were as follows: barium carbonate 90% enriched with ^{13}C, Mound Laboratory (Monsanto); cation-exchange resin, Dowex AG-50W-X4, 100–200 mesh, Bio-Rad; Sepharose 4B and SP-Sephadex C-25, Pharmacia; N^{α}-acetyl-L-arginine methyl ester, Vega-Fox (used without purification); *N,N*-dimethylformamide, *N,N*-dimethylformamide diethyl acetal, protopine, and cryptopine, Aldrich; Pipes, Sigma (114C-5024 and 27C-5044); hippuryl-L-arginine, Sigma (45C-5057) and Cyclo Chemicals (951023); *p*-nitrophenyl *p*-guanidobenzoate, Nutritional Biochemicals Corp. (5997); $[^{13}C^{\circ}]$-DL-arginine (90% isotope enrichment), Prochem. All other chemicals were reagent grade or the highest available grade.

Synthesis of $[^{13}C]$*-O-Methyl Isourea.* Carbon-13-labeled *O*-methyl isourea was prepared from $Ba^{13}CO_3$ (Turba & Leismann, 1953). Dry $Ba^{13}CO_3$ was reacted with NH_3 for 8 h in a tube furnace at 760 °C. The product, $Ba(^{13}CN)_2$, was converted to $Ag_2(^{13}CN)_2$ by treatment with 2 M NH_4OH

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[2] Hunkapiller et al. (1979) reported results of a ^{13}C NMR study of $[^{13}C^{\circ}]$Arg incorporated into STI–trypsin(?) by the method of Sealock and Laskowski (1969). Experimental details were not given, nor was characterization of the product described. They apparently used the original product of the enzymatic semisynthesis step, the STI–trypsin(?) complex, without dissociation of the complex and purification of the semisynthetic inhibitor. This was an essential step of the Sealock and Laskowski (1969) procedure. Work from this laboratory indicates that this material is highly degraded and differs electrophoretically from STI complexes with either bovine α- or β-trypsin (Baillargeon, 1980). Hence, we do not presume to interpret their results except to point out that (1) the extra peak they observe at pH 7.1 at 173.3 ppm and assign to a covalent acyl STI–trypsin complex is probably an impurity, (2) the peak they observe at pH 2.9 at 177.5 ppm and assign to STI(63,64) cannot correspond to STI(63,64), and (3) the major point of their experiment is in agreement with ours, nonetheless, in that there is no major upfield chemical shift of the Arg^{63} C° in the complex with trypsin.

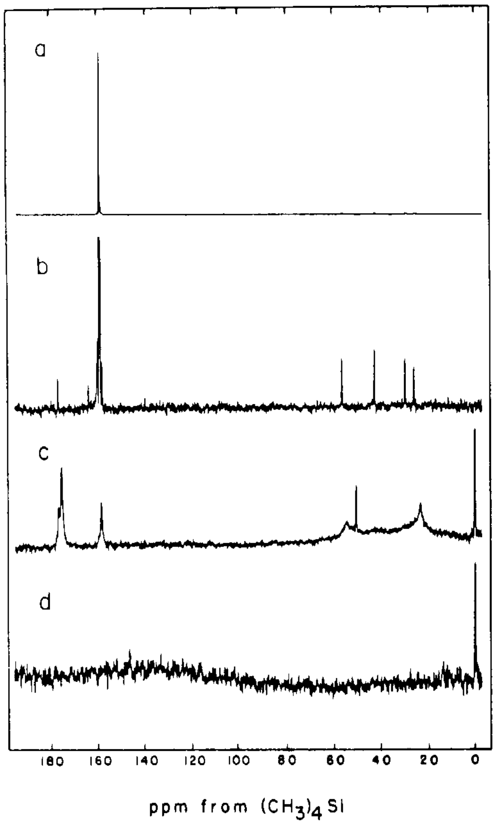

FIGURE 2: ^{13}C NMR spectra of model compounds at 20 MHz. (a) 90% [$^{13}C^{\zeta}$]-L-arginine used in the protein incorporation studies. (b) Expanded scale of spectrum a showing the natural-abundance carbons of arginine (the ^{13}C-enriched C^{ζ} is off scale). The resonance at 163.8 ppm was determined to be an impurity. The symmetrical peaks around the central guanido peak are spinning side bands. (c) N^{α}-acetyl-L-arginine methyl ester bound to AG-50W-X4 cation-exchange resin at neutral pH*. (d) Spectrum of AG-50W-X4 cation-exchange resin in ^2H_2O. The peak at 0 ppm in (c) and (d) corresponds to the external reference, $(CH_3)_4Si$. Spectral conditions: 2000–4000 pulses; 90° pulse width (14 μs); 3-s delay between pulses; 8K data points; 30 °C; broad band ^1H decoupling.

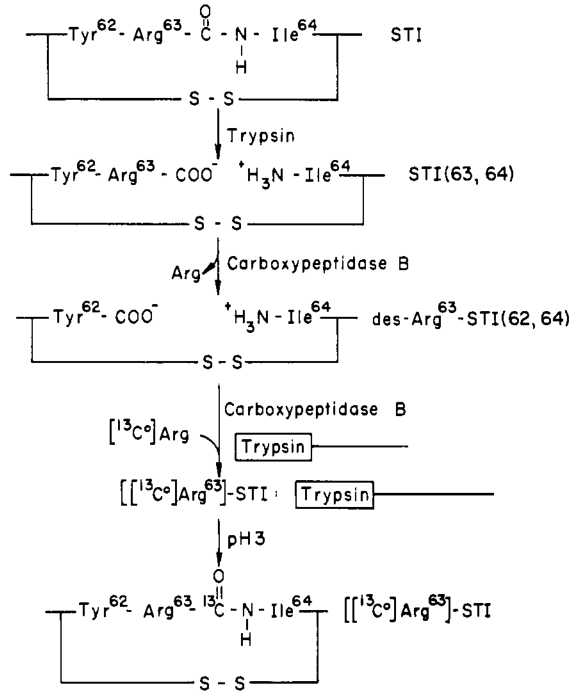

FIGURE 3: Semisynthesis flow chart: R is $-(CH_2)_3NHC(NH)NH_2$ and $\boxed{\text{trypsin}}$ represents insoluble trypsin. For experimental details, see Sealock & Laskowski (1969) and the text.

and excess $AgNO_3$. The $Ag_2(^{13}CN)_2$ was filtered, dried, and reacted with methanolic HCl for 5 days (Stieglitz & McKee, 1900). AgCl was filtered off, and the [^{13}C]-*O*-methyl isourea was recovered by evaporating the methanol and crystallizing the resulting residue from methanol by the addition of dry ethyl ether. The purity was checked by NMR spectroscopy, and the overall yield based on $Ba^{13}CO_3$ was typically 90–95%.

Synthesis of [$^{13}C^{\zeta}$]-*L-Arginine.* L-Ornithine was guanidinated with [^{13}C]-*O*-methyl isourea (Kurtz, 1949). A twofold excess of ornithine was used to ensure maximal reaction with the [^{13}C]-*O*-methyl isourea. Arginine was isolated from the reaction mixture as the flavianate complex and was recovered as described by Kurtz (1949), except that aniline was used instead of pyridine to neutralize the excess hydrochloric acid prior to crystallization (Cox, 1928). Purity of the product was checked by ^{13}C NMR (Figure 2a,b) and ^1H NMR spectroscopy and by amino acid analysis. Typical yields were 60–80% based on *O*-methyl isourea.

Preparation of [$^{13}C^{\circ}$]-*L-Arginine.* [$^{13}C^{\circ}$]-DL-Arginine was tested for purity by amino acid analysis and ^{13}C NMR spectroscopy and purified by passage through a Chelex column. It was necessary to resolve the [$^{13}C^{\circ}$]-DL-arginine for the enzymatic semisynthetic procedure, since carboxypeptidase B is inhibited by the high concentrations of arginine used. The DL mixture was acetylated, and the L isomer was hydrolyzed selectively by using porcine acylase I (Greenstein & Winitz, 1961) and purified by ion-exchange chromatography on Dowex AG-50W-X4. It was found that the success of this procedure depended on prior removal of a copper ion impurity present in the commercial [$^{13}C^{\circ}$]-DL-arginine which completely inhibited acylase I. [Cu(II) normally is used to mask the α-amino group of ornithine when it is guanidinated (Kurtz, 1949) to yield arginine.] The purity of the [$^{13}C^{\circ}$]-L-arginine product was established by amino acid analysis, and the degree of ^{13}C enrichment was determined by ^{13}C NMR spectroscopy.

Preparation of [[^{13}C]*Arg*^{63}]*STI.* The method of Sealock & Laskowski (1969) was modified for large-scale preparation of [Lys^{63}]STI or [Arg^{63}]STI products through the use of insolubilized trypsin, which permits one-step purification and recovery of STI products and recycling of excess reactants and catalyst (see Figure 3). The insoluble trypsin was formed by adding bovine trypsin to Sepharose 4B previously activated with cyanogen bromide (March et al., 1974); it typically bound 0.8–2 mg of STI/mL bed volume. It should be noted that the use of insoluble trypsin is not feasible when the added P_1 amino acid causes the product to bind slowly to trypsin. In such cases, formation of the complex between insoluble trypsin and STI(63,64) becomes rate limiting, rather than incorporation of the P_1 amino acid into des-Arg^{63}-STI(62,64) by carboxypeptidase B (Kowalski et al., 1974).

A solution of porcine carboxypeptidase B, ^{13}C-labeled arginine, and des-Arg^{63}-STI(62,64) in pH 6.7 buffer containing 0.05 M Pipes, 0.14 M KCl, and 0.03 M $CaCl_2$ was circulated through an insoluble trypsin column of 3–40-mL bed volume at a flow rate of 1 mL/min for 1 week. Typical concentrations were 0.1 M ^{13}C-labeled arginine, 3×10^{-4} M des-Arg^{63}-STI(62,64), and 1×10^{-4} M carboxypeptidase B. Under these conditions, the yield is limited by the STI-binding capacity of the insoluble trypsin. The activity of carboxypeptidase B was determined by using hippuryl-L-arginine (Wolff et al., 1962).

The reactions were terminated by eluting the column with pH 6.7 buffer containing 0.05 M Pipes, 0.14 M KCl, and 0.03 M $CaCl_2$ until the absorbance fell to 0.03 ODU at 280 nm;

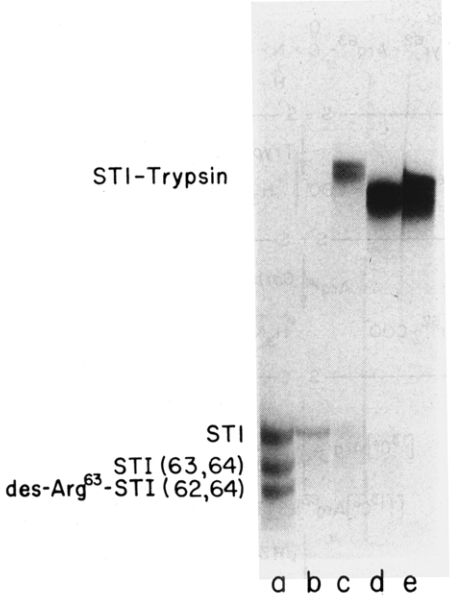

FIGURE 4: Gel characterization of $[[^{13}C^{\circ}]Arg^{63}]STI$: (a) STI controls; (b) $[[^{13}C^{\circ}]Arg^{63}]STI$; (c) $[[^{13}C^{\circ}]Arg^{63}]STI$ with 50 mol % trypsin; (d) $[[^{13}C^{\circ}]Arg^{63}]STI$ with 90 mol % trypsin; (e) $[[^{13}C^{\circ}]Arg^{63}]STI$ with excess trypsin after NMR experiments.

the fractions were saved for later recovery of excess reactants. The insoluble trypsin column was then washed with 0.2 M glycine, pH 2, until the eluant absorbance fell to 0.03 ODU at 280 nm. The STI-containing fractions were dialyzed against H_2O and lyophilized. Yields of $[[^{13}C]Arg^{63}]STI$ equal to 40–65% of the binding capacity of the insoluble trypsin column were obtained. During the time required to elute the STI from the STI–trypsin column, some STI was converted to modified STI; the recovered protein was 90% STI and 10% STI(63,64). Since the recovered STI was eluted from an insoluble trypsin column, it was known to be active inhibitor. Also, addition of excess trypsin to the recovered STI caused the formation of an STI–trypsin complex band and the concurrent disappearance of inhibitor bands as assayed by disc gel electrophoresis (Kowalski & Laskowski, 1972). A typical example is shown in Figure 4.

Recovery of Excess Reactants. Ultrafiltration with an Amicon UM-10 membrane was used to separate unreacted $[^{13}C]$-L-arginine and to concentrate the excess des-Arg^{63}-STI(62,64) and carboxypeptidase B. By this method, up to 75% of the initial carboxypeptidase B activity could be recovered and reused.

Preparation of Complexes. The STI–trypsin complexes were prepared by adding trypsin to STI. The concentration of trypsin was determined by active site titration with *p*-nitrophenyl *p*-guanidobenzoate (Chase & Shaw, 1967) and by the rate of reaction with the substrate N^{α}-benzoyl-DL-arginine-4-nitroanilide (Erlanger et al., 1961). The concentration of STI was determined by titration with trypsin of known concentration. The fraction of STI–trypsin complex present also was measured by using disc gel electrophoresis. The following empirical staining factor was determined, where A refers to the integrated areas of the respective peaks in the densitometer scan:

$$\text{fraction complex} = A_{\text{complex}}/(1.92\, A_{\text{STI}} + A_{\text{complex}})$$

Preparation of $[[^{13}C^{\circ}]Arg^{63}]STI(63,64)$. Modified STI was obtained from the STI–trypsin complex by ion-exchange chromatography on SP-Sephadex at pH 2.5 (Barnhill & Trowbridge, 1975). The recovered material contained 70% STI(63,64) and 30% STI by disc gel electrophoresis.

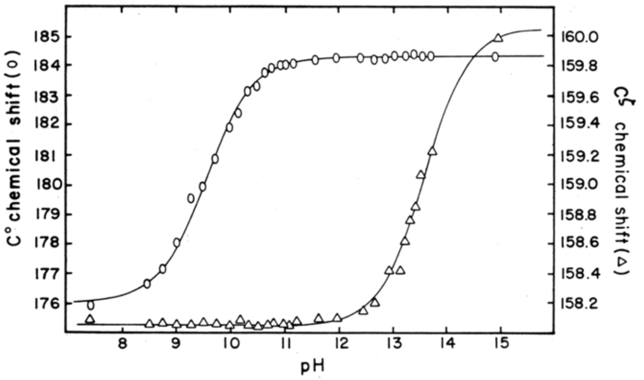

FIGURE 5: 20-MHz ^{13}C NMR pH titration curves of the C° (○) and C^{ζ} (△) carbons of L-arginine (0.1 M) in ^2H_2O. Arginine hydroxide was titrated with 1.0 M ^2HCl and KO^2H. The curves were determined by using a nonlinear least-squares analysis computer program. The fitted pK_a' values are given in the text.

Titration and pH Measurement. All pH adjustments and measurements were made in 5-mL glass reaction vials (Pierce) using methods described previously (Markley & Porubcan, 1976). The L-arginine samples used for pH titration studies were degassed by bubbling N_2 through the sample before and after determining the pH*. The pH* was determined again after obtaining the chemical shift, and the results were discarded if the pH* had changed more than 0.1 pH unit. Glass electrode pH measurements become inaccurate above pH 13.7. To obtain an end point, we dissolved L-arginine hydroxide in 1.0 M KO^2H and assigned the pH by calculation. The data for both the C° and C^{ζ} carbons were then curve fitted by using a nonlinear least-squares analysis computer program (W. R. Finkenstadt and D. E. Neves, unpublished experiments).

NMR Spectroscopy. ^{13}C NMR spectra were obtained at 25.14 MHz by using a Varian XL-100 with a home-built Fourier-transform modification based on a Nicolet 1080 computer, at 20 MHz using a Varian CFT-20, at 37.7 MHz using a Nicolet NT-150, or at 90.55 MHz using a Nicolet NT-360 spectrometer. All protein spectra were taken at a probe temperature of 30 °C, maintained by a variable temperature unit. Chemical shifts are reported relative to external $(CH_3)_4Si$. Chemical shifts were measured by inserting a capillary of either $(CH_3)_4Si$, CS_2, or 40% dioxane in water into the NMR tube containing the sample of interest. In the case of CS_2, chemical shifts were then converted to the $(CH_3)_4Si$ reference by using the equation $\delta_{(CH_3)_4}Si = 193.7 - \delta_{CS_2}$. Dioxane chemical shifts were translated to the $(CH_3)_4Si$ reference by assuming a dioxane chemical shift of 67.8 ppm. Pipes buffer peaks were used as an internal standard.

Results

Model Compounds. In Figure 5 are shown pH titration data for the C° and C^{ζ} of L-arginine in ^2H_2O. The data indicate that a −2.0-ppm (upfield) protonation shift occurs at the arginine C^{ζ}. The pK_a' obtained for C^{ζ} is 13.6 ± 0.2. The titration shift of the C° with a pK_a' of 9.56 ± 0.03 is due to the protonation of the α-amino group.

The Arg^{63} C^{ζ} ion pairs with the carboxylate of Asp$^{189'}$ in STI–trypsin (Sweet et al., 1974). An attempt was made to simulate such an interaction by obtaining ^{13}C NMR spectra of an arginine derivative bound to a cation-exchange resin (Sternlicht et al., 1971). N^{α}-Acetyl-L-arginine methyl ester (AcArgOMe) was used instead of L-arginine to ensure that the guanidinium group rather than the amino group bound to the resin. AcArgOMe in ^2H_2O was passed through a column (1.5-mL bed volume) of Dowex AG-50W-X4 cat-

Table I: Data for Compounds Used to Model the ^{13}C NMR Chemical Shift of a Carbonyl Undergoing Partial Nucleophilic Attack

species	N–C distance, d (Å)	d^{-1} (Å^{-1})	δ_{raw}	δ_{cor}
3-pentanone	∞	0		211.4[g]
cryptopine	2.581[a]	0.388	196.39	210.3[h]
protopine	2.555[b]	0.391	194.82	208.7[h]
clivorine	1.993[c]	0.502	193.07	202.2[i]
retusamine	1.64[d]	0.609	188.3[f]	197.4[i]
N,*N*-dimethylformamide diethyl acetal	1.48[e]	0.676	110.6	117[j]

[a] Hall & Ahmed (1968b). [b] Hall & Ahmed (1968a). [c] Birnbaum (1972). [d] Wunderlich (1967). [e] Covalent bond distance (Sutton, 1965). [f] C. C. J. Culvenor, personal communication. [g] Chemical shift from Johnson & Jankowski (1972). [h] 13.9-ppm correction for an α-phenoxy group. [i] 9.1-ppm correction for an α-vinyl group. [j] See the text for correction procedure.

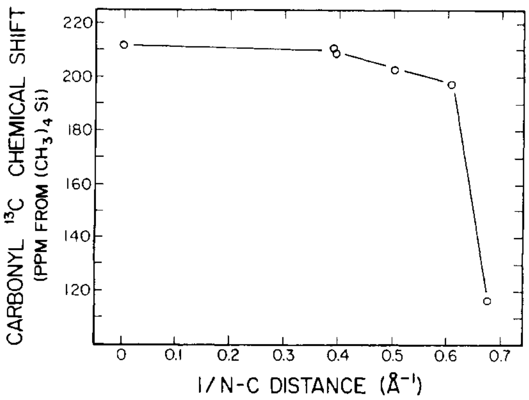

FIGURE 6: Plot of the corrected chemical shift of the carbonyl carbon undergoing nucleophilic addition as a function of the reciprocal of the N–C distance. Data are from Table I. The line has no theoretical significance.

Table II: Experimental Chemical Shifts of Arginines

species	arginyl chemical shift[a]	
	$^{13}C^{\zeta}$	$^{13}C^{\circ}$
[$^{13}C^{\zeta}$]-L-arginine (^1H_2O or ^2H_2O, pH* 7.0)	158.05 ± 0.04	
N^{α}-acetyl-L-arginine methyl ester[b]	158.00 ± 0.25	
[[$^{13}C^{\zeta}$]Arg^{63}]STI (^1H_2O or ^2H_2O, pH* 7.0)	157.81 ± 0.02	
[[$^{13}C^{\zeta}$]Arg^{63}]STI–trypsin (^1H_2O, pH 7.0)[c]	158.04 ± 0.02	
[$^{13}C^{\circ}$]-L-arginine (pH* 7.0)		175.8 ± 0.1
H-Gly-Arg-Ala-OH (neutral pH)		173.4 ± 0.1[d]
H-Phe-Pro-Arg-OH (neutral pH)		179.1 ± 0.1[e]
[[$^{13}C^{\circ}$]Arg^{63}]STI (pH* 7.0)		173.8 ± 0.1
[[$^{13}C^{\circ}$]Arg^{63}]STI(63,64) (pH(7.0)		179.0 ± 0.1
[[$^{13}C^{\circ}$]Arg^{63}]STI(63,64) (pH* 2.9)		174.7 ± 0.1
[[$^{13}C^{\circ}$]Arg^{63}]STI–trypsin (pH* 7.0)[f]		174.7 ± 0.1

[a] Chemical shift in ppm relative to external $(CH_3)_4Si$. [b] Bound to AG-50W-X4 cation-exchange resin at neutral pH. [c] Porcine or bovine trypsin. [d] Chemical shift from Richarz & Wüthrich (1978). [e] Chemical shift from Christl & Roberts (1972). [f] Bovine trypsin.

ion-exchange resin. The column was washed thoroughly with ^2H_2O to remove excess AcArgOMe. The resin with bound AcArgOMe was then transferred to an NMR tube for the ^{13}C NMR experiment. The results of this experiment and the control experiment using only the resin are shown in parts c and d of Figure 2. Because of their low mobilities (Sternlicht et al., 1971), all the resin carbons are too broad to be distinguishable, as are the protonated side-chain carbons of the bound arginine derivative. The peaks at 23.09 and 50.16 ppm correspond to the acetyl and methyl groups, respectively, of resin-bound AcArgOMe; the chemical shift of the Arg C^{ζ} (158.00 ppm) is nearly identical with that for free arginine (158.05 ppm), indicating that formation of a guanidinium–sulfonic acid ion pair has almost no influence on the guanido carbon chemical shift.

We now turn to models for [[$^{13}C^{\circ}$]Arg^{63}]STI. In order to quantitate the chemical shift expected for *partial* nucleophilic attack of the kind proposed by Huber et al. (1974, 1975) for PTI–trypsin, we obtained ^{13}C NMR chemical shifts for members of the series of alkaloids described by Bürgi et al. (1973). Each of these compounds contains a carbonyl group that is undergoing partial transannular nucleophilic attack by a tertiary aliphatic amine. The nitrogen–carbonyl carbon distance, *d*, for each species in the crystal is known from X-ray diffraction. The raw chemical shifts, δ_{raw}, of these compounds in deuteriochloroform are listed in Table I. These have been converted by standard additivity corrections (Wehrli & Wirthlin, 1976) to the chemical shifts, δ_{cor}, expected of 3-pentanone undergoing similar nucleophilic attack (Table I). The δ_{cor} values are plotted as a function of d^{-1} in Figure 6. Unless the N–C bond distances of the alkaloids are much longer in chloroform solution than in crystals, the results indicate that the carbonyl carbon chemical shift is relatively insensitive to partial nucleophilic attack. The largest chemical shift change occurs between d = 1.60 and 1.48 Å. Thus, a contribution of 0 to −5 ppm is expected for the proteinase–inhibitor structure proposed by Huber et al. (1974, 1975) where the C°–O distance is 2.6 Å.

The chemical shift of the [$^{13}C^{\circ}$]Arg^{63} in STI may be compared to the arginyl C° of model peptides such as H-Gly-Gly-Arg-Ala-OH (173.4 ppm) (Richarz & Wüthrich, 1978). The chemical shift of the tetrahedral intermediate in peptide hydrolysis is estimated to be 118 ± 10 ppm based on the following two models. (1) The chemical shifts of *N*,*N*-dimethylformamide and *N*,*N*-dimethylformamide diethyl acetal in deuteriochloroform are 162.4 and 110.6 ppm, respectively. The acetal differs from the tetrahedral intermediate in peptide hydrolysis by replacement of the hydrogen atom with an alkyl group, a change of +6.3 ppm based on the carbonyl shifts of acetaldehyde and acetone (Johnson and Jankowski, 1972), resulting in an estimated shift of 117 ppm; (2) The tetrahedral carbon of hemiorthoesters, R*C(OH)(OR)$_2$, which contain three electronegative substituents (three oxygens rather than two oxygens and one nitrogen as present in the proposed tetrahedral intermediate in peptide hydrolysis) has a shift around 119 ppm (Hänni et al., 1975). Hence, one anticipates an upfield shift of about 173 ppm − 118 ± 10 ppm = 55 ± 10 ppm on production of a covalent tetrahedral species of the kind predicted by Sweet et al. (1974) for STI–trypsin.

[[$^{13}C^{\zeta}$]Arg^{63}]STI and [[$^{13}C^{\zeta}$]Arg^{63}]STI–Trypsin. The ^{13}C NMR spectrum of unenriched STI (Figure 7a) contains an envelope of natural-abundance peptide carbonyl resonances in the region 170–180 ppm. The spectrum of [[$^{13}C^{\zeta}$]Arg^{63}]STI (Figure 7b) shows an additional strong resonance at 157.81 ppm which we assign to the guanido carbon of Arg^{63}. The peaks between 50 and 60 ppm are from Pipes buffer used in the preparation of ^{13}C-labeled STI. The chemical shift of the labeled carbon is the same in both ^2H_2O and ^1H_2O (Table II) and is 0.24 ppm upfield from its position in free arginine.

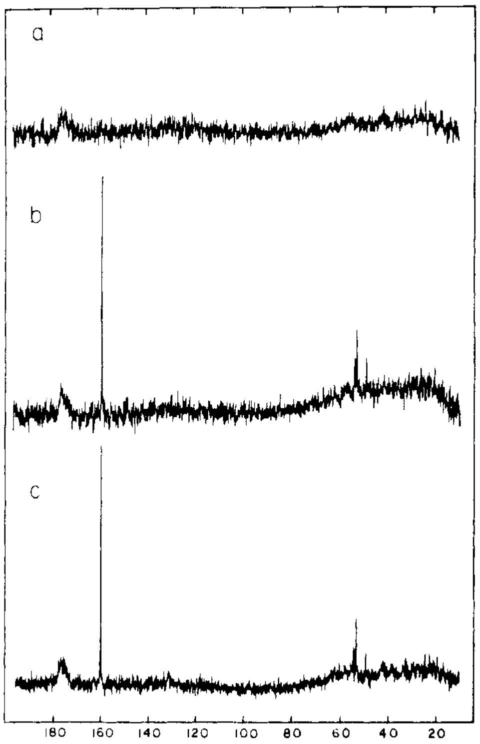

FIGURE 7: ^{13}C NMR spectra of STI and the STI–trypsin complex at 20 MHz. Samples consisted of 30 mg of STI or 50 mg of STI–trypsin (porcine) complex in 0.8 mL of water containing 0.5 M KCl, pH 7.0, in 8-mm flat-bottomed NMR tubes, 30 °C. Spectral conditions: broad band ^1H decoupling; 8K data points; 2.5-Hz exponential weighting factor. (a) Unenriched (natural-abundance) STI in ^2H_2O [12 000 pulses; 90° pulse width (14 μs); 7.5 s between pulses]. (b) STI ^{13}C enriched at the C^{ζ} of Arg^{63}, ^1H_2O. The peaks between 50 and 58 ppm are from Pipes buffer used in sample preparation [10 000 pulses; 90° pulse width (14 μs); 7.5 s between pulses]. (c) STI–trypsin complex with the STI ^{13}C enriched at the C^{ζ} of Arg^{63}, ^1H_2O. The peaks between 50 and 58 ppm are from Pipes buffer [26 390 pulses; 45° pulse width (7 μs); 2.0 s pulses].

An equimolar amount of $[^{13}C^{\zeta}]$-L-arginine was added to a solution of $[[^{13}C^{\zeta}]Arg^{63}]$STI in order to test the possibility that the peak at 157.81 ppm arises from contaminating $[^{13}C^{\zeta}]$arginine rather than from the protein. An additional sharper peak appeared at low field. Since the chemical shift of the additional peak was slightly different from that of free arginine, this may indicate that L-arginine binds to STI.

The ^{13}C NMR spectrum of $[[^{13}C^{\zeta}]Arg^{63}]$STI–porcine trypsin (Figure 7c) shows resonances from Pipes buffer between 50 and 60 ppm, the enriched guanido carbon at 158.04 ppm, and the natural-abundance C° peaks between 170 and 180 ppm. An equivalent chemical shift was found for the complex between $[[^{13}C^{\zeta}]Arg^{63}]$STI and bovine trypsin. The chemical shift of the guanido carbon in STI–trypsin happens to be the same (within experimental error) as that measured for free arginine.

The Arg^{63} C^{ζ} peak of STI is broader (at 20 MHz) in ^1H_2O (2.6 ± 0.4 Hz) than in ^2H_2O (1.3 ± 0.2 Hz) as predicted by theory (Allerhand, 1979). However, the Arg^{63} C^{ζ} peak is narrower (at 20 MHz) in the trypsin complex than in free STI (1.5 ± 0.2 Hz in ^1H_2O). It is possible that hydrogen-bond donation by the guanido group in the trypsin specificity pocket results in narrowing of the guanido carbon resonance. The arginine C^{ζ} is broadened when AcArgOMe binds to sulfonic acid cation-exchange resin (Figure 2). However, the motion of the resin-bound guanido carbon is highly anisotropic (Sternlicht et al., 1971), and comparison of the line width of resin-bound arginine with that of the STI–trypsin complex is not appropriate.

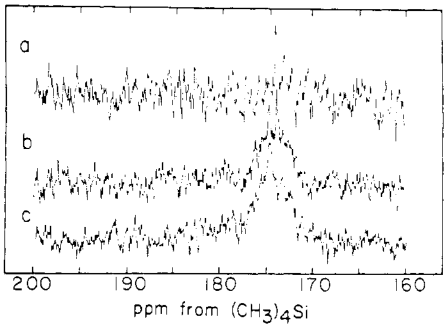

FIGURE 8: ^{13}C NMR spectra at 90.55 MHz of the C° region of (a) $[[^{13}C^{\circ}]Arg^{63}]$STI (67 396 pulses), (b) $[[^{13}C^{\circ}]Arg^{63}]$STI–bovine trypsin, 2:1 stoichiometry (159 744 pulses), and (c) $[[^{13}C^{\circ}]Arg^{63}]$-STI–bovine trypsin, 1:1 stoichiometry (153 852 pulses). All samples were at pH* 7.0 in ^2H_2O containing 0.1 M KCl. Spectral conditions: 16K data points; 0.45-s delay between pulses; 4-μs pulse width (25 μs = 90° pulse); 10-Hz exponential weighting factor; 30 °C; broad band ^1H decoupling.

$[[^{13}C^{\circ}]Arg^{63}]$STI and $[[^{13}C^{\circ}]Arg^{63}]$STI–Trypsin. The chemical shift of $[[^{13}C^{\circ}]Arg^{63}]$STI is 173.8 ppm (Figure 8a), or −2.0 ppm (upfield) from the C° peak of free arginine. When a half-stoichiometric amount of trypsin was added, two peaks appeared in the spectrum with chemical shifts 173.8 and 174.7 ppm as shown in Figure 8b. Finally, when a full stoichiometric amount of trypsin was added (Figure 8c), only a single peak remained at 174.7 ppm (Table II). Disc gel electrophoretic analyses of the samples used for NMR spectroscopy are shown in Figure 4.

Upon recovery of the $[[^{13}C^{\circ}]Arg^{63}]$STI from the complex after the NMR experiments, the sample contained more than 97% native STI. No evidence of nonspecific bond cleavage was found by NH_2-terminal amino acid sequencing (with a Beckman sequencer) or by disc gel electrophoresis.

$[[^{13}C^{\circ}]Arg^{63}]$STI(63,64). ^{13}C NMR spectra were obtained with modified STI prepared from $[[^{13}C^{\circ}]Arg^{63}]$STI. The ^{13}C NMR chemical shift of the labeled carbon is expected to be different at the two pH values studied (pH* 7.0 and pH* 2.9) because of protonation of the Arg^{63} carboxylate which has a pK_a' of 3.56 (Mattis & Laskowski, 1973). Christl & Roberts (1972) obtained a C° protonation shift of −3.5 ± 0.2 ppm for the carboxyl terminal of H-Phe-Arg-OH. The chemical shift of $[[^{13}C^{\circ}]Arg^{63}]$STI(63,64) is 179.0 ± 0.1 at pH 7.0 and 174.7 ± 0.1 at pH 2.9. Hence, the protonation shift is −4.3 ± 0.2 ppm. The arginine carboxylate chemical shift of STI(63,64) is equivalent to that of the model peptide H-Phe-Pro-Arg-OH at neutral pH (Christl & Roberts, 1972; Table II).

Discussion

When L-arginine is incorporated into position 63 of STI, the C^{ζ} shifts −0.24 ppm (upfield); upon subsequent formation of the STI–trypsin complex, the Arg^{63} C^{ζ} shifts +0.23 ppm (downfield). These environmental shifts are comparable to those reported by Oldfield et al. (1975) for several small proteins but are smaller than some found recently for $[^{13}C^{\eta}]$-L-homoarginine and $[^{13}C^{\zeta}]$-L-arginine in other proteins (Stellwagen et al., 1977; Cocco et al., 1977). In the latter studies, a paramagnetic heme group (Stellwagen et al., 1977) or a large aromatic nucleotide ligand (Cocco et al., 1977) was present. We suggest that the small upfield chemical shift of Arg^{63} C^{ζ} in STI may be due to ring current interactions with the adjacent Tyr^{62}.

Sweet et al. (1974) found in their X-ray crystallographic study of the STI–trypsin complex that the guanidinium group of Arg^{63} of STI ion pairs with the carboxylate of Asp$^{189'}$ of trypsin. The deprotonation shift of the Arg^{63} C$^{\zeta}$ is +2 ppm (downfield) (Figure 5). Since the Arg^{63} C$^{\zeta}$ of STI shifts only +0.23 ppm (downfield) upon complexation with trypsin, there apparently is little if any proton transfer from the guanidinium group to the carboxylate of Asp$^{189'}$. Similar reasoning may be used to rule out proton transfer in the complex between N^{α}-acetyl-L-arginine and sulfonate resin (Figure 5).

Our ^{13}C NMR experiments with [[^{13}C$^{\circ}$]Arg^{63}]STI show a small (0.9 ppm) downfield shift of the labeled carbon upon complexation of the inhibitor with trypsin. Similar results were obtained by Richarz et al. (1980) in the analogous experiment with PTI, in which [^{13}C$^{\circ}$]Lys was incorporated into the P$_1$ reactive site position by enzymatic semisynthesis. The chemical shift of the Lys^{15} C$^{\circ}$ was found to be 175.7 ppm in the free inhibitor and 176.4 ppm in its complexes with either trypsin or anhydrotrypsin (Richarz et al., 1980). These results provide further proof that the mechanisms of interaction of the two evolutionarily unrelated inhibitors with trypsin are essentially identical (Laskowski & Kato, 1980).

The NMR results rule out the covalent structure proposed (Sweet et al., 1974) for STI–trypsin, since the C$^{\circ}$ of residue P$_1$ in STI shifts downfield by 0.9 ppm on complex formation. As discussed above, tetrahedral bond formation is expected to lead to an upfield shift of ~55 ppm. Stronger hydrogen-bond donation to the C$^{\circ}$-oxygen of residue P$_1$ in the complex (Robertus et al., 1972) as compared to the free inhibitor in water should result in a downfield shift (Maciel & Ruben, 1963); however, the shift is expected to be less than 5 ppm (Llinás et al., 1977a). Although the data indicate that the inhibitor–proteinase complex (species C, eq 1) is not a covalent, fully tetrahedral adduct, this does not imply the absence of tetrahedral intermediates elsewhere in the catalytic mechanism.

The present NMR data alone cannot rule out a complex that is an acyl–enzyme intermediate, since the chemical shifts of an amino acid C$^{\circ}$ in a peptide or ester linkage are similar and within the uncertainty of environmental shifts. However, the acyl structure can be eliminated on the grounds that anhydrotrypsin also forms species C (eq 1) (Ako et al., 1974; Huber et al., 1975). In spite of the absence of the Ser$^{195'}$ hydroxyl, the reactive site P$_1$ C$^{\circ}$ is 50% tetrahedrally distorted in the PTI–anhydrotrypsin complex, just as it is in PTI–trypsin (Huber & Bode, 1978; see Figure 1). Although covalent bond formation is impossible, anhydrotrypsin reacts with PTI and PTI(15,16) and with STI and STI(63,64) to form complexes that appear identical with species C and X of eq 1, respectively (G. H. Widener and M. Laskowski, Jr., unpublished experiments). Mechanisms of amide hydrolysis consistent with eq 1 have been proposed by Satterthwait & Jencks (1974; mechanism 2) and by Komiyama & Bender (1979).

It is not possible on the basis of present ^{13}C NMR results to distinguish between a complex with a trigonal C$^{\circ}$ at the reactive site P$_1$ position and the structure proposed for PTI–trypsin (Huber et al., 1974, 1975; Huber & Bode, 1978) which involves incipient nucleophilic addition at C$^{\circ}$, hydrogen-bond donation to the C$^{\circ}$-oxygen, and distortion of the C$^{\circ}$-oxygen out of the peptide plane. The model studies discussed above (Table I; Figure 6) suggest that 0 to −5 ppm (upfield) may be a good estimate for the contribution from a 2.6 Å approach of the Ser$^{195'}$ oxygen. However, this can be narrowed to less that 0.1 ppm upfield on the basis of the experiments discussed in the following paper (Richarz et al., 1980) in which the chemical shift of the C$^{\circ}$ of residue P$_1$ of PTI was determined for the complex with trypsin and anhydrotrypsin. The more obvious contributors to the observed small downfield shift on complex formation are hydrogen bonding and out of plane distortion of the C$^{\circ}$-oxygen. Although adequate model studies of peptide carbonyl deformation are not available, studies of cyclic peptides (Grathwohl et al., 1975; Llinás et al., 1976, 1977b) indicated that downfield shifts of up to 2 ppm arise from distortions of planar peptide bonds through steric strain. We expect that peptide carbonyl deformation in the trypsin inhibitor complexes could lead to a downfield shift of similar magnitude. As discussed above, hydrogen bonding of the C$^{\circ}$-oxygen could lead to an additional but small downfield shift.

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