Carbon-13 Nuclear Magnetic Resonance Studies of the Selectively Isotope-Labeled Reactive Site Peptide Bond of the Basic Pancreatic Trypsin Inhibitor in the Complexes with Trypsin, Trypsinogen, and Anhydrotrypsin†

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ABSTRACT: A previously characterized modification of the basic pancreatic trypsin inhibitor (BPTI), with the carbonyl carbon atom of Lys-15 selectively enriched in ^{13}C, the peptide bond Arg-39–Ala-40 cleaved, and Arg-39 removed, was used for ^{13}C NMR studies of the reactive site peptide bond Lys-15–Ala-16 in the complexes with trypsin, trypsinogen, and anhydrotrypsin. The chemical shift of [1-^{13}C]Lys-15 was 175.7 ppm in the free inhibitor, 176.4 ppm in the complexes with trypsin and anhydrotrypsin and the ternary complex with trypsinogen and H-Ile-Val-OH, and 175.7 ppm in a neutral solution containing the inhibitor and trypsinogen. These data show that the trypsin–BPTI complex does not contain a covalent tetrahedral carbon atom in the position of the reactive site peptide carbonyl of the inhibitor. They would be consistent with the formation of a noncovalent complex but cannot at present be used to further characterize the degree of a possible pyramidalization of the carbonyl carbon of Lys-15 in such a complex. The identical chemical shifts in the complexes with trypsin and anhydrotrypsin indicate that the γ-hydroxyl group of Ser-195 of trypsin does not have an important role in the binding of the inhibitor. The previously described [Perkins, S. J., & Wüthrich, K. (1980) *J. Mol. Biol. 138*, 43–64] stepwise transition from the trypsinogen conformation to an intermediate conformational state in the trypsinogen–BPTI complex and a trypsin-like conformation in the ternary complex trypsinogen–BPTI–H-Ile-Val-OH appears to be manifested also in the chemical shift of [1-^{13}C]Lys-15 of labeled BPTI.

Protein–protein interactions in the complexes formed between proteinases and protein proteinase inhibitors have been intensively investigated by a variety of methods (Means et al., 1974; Sweet et al., 1974; Blow et al., 1974; Vincent et al., 1974; Tschesche, 1974; Huber & Bode, 1978). A fundamental question in these studies concerned the state of the reactive site peptide bond of the inhibitor in the complexes with the enzymes. In the early interpretations of crystallographic data, it was concluded that the carbonyl carbon of the reactive site peptide bond in the enzyme-bound inhibitor adopts a covalent tetrahedral configuration (Rühlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974). Subsequently, on the basis of refined X-ray data, the formation of a covalent tetrahedral carbon was ruled out and it was concluded that a noncovalent complex was formed where the carbonyl carbon of Lys-15 assumed a tetrahedrally distorted structure intermediate between sp^2 and sp^3 hybridization and that the γ-oxygen atom of Ser-195 from trypsin was at a distance of 2.6 Å from this carbonyl carbon (Huber et al., 1974; Huber & Bode, 1978). Alternatively, it was also suggested that inhibitor binding by the protease involved formation of an acyl–enzyme intermediate (Hixson & Laskowski, 1970). The present paper reports on the use of a selectively ^{13}C-enriched modification of BPTI[1] for ^{13}C NMR studies of the reactive site carbonyl carbon in the enzyme-bound inhibitor.

The preparation of the labeled BPTI used for these studies was described previously (Richarz et al., 1979). The structural characterization by NMR and chemical methods showed that the reactive site peptide bond Lys-15–Ala-16 was enriched in ^{13}C and that the peptide bond Arg-39–Ala-40 was cleaved and Arg-39 removed during the enzymatic modification reactions. The spatial structure of native BPTI was preserved in this labeled inhibitor, which also had full inhibitory activity toward trypsin and thus seemed a suitable compound for ^{13}C NMR studies of enzyme–inhibitor interactions (Richarz et al., 1979).

In addition to the trypsin complex, the interactions of labeled BPTI with anhydrotrypsin and trypsinogen were also studied. Anhydrotrypsin has the catalytic site Ser-195 converted to dehydroalanine. Anhydrotrypsin prepared according to Ako et al. (1974) is enzymatically inactive but binds BPTI with nearly the same strength as trypsin (Vincent et al., 1974; Ako et al., 1974). Comparison of the complexes with trypsin and anhydrotrypsin should provide a way to assess the role of direct interactions of the inhibitor with the hydroxyl group of the reactive site serine in the interactions between the two proteins. The study was further extended to include trypsinogen interactions with the labeled inhibitor, mainly in the hope to gain additional insights into the structural transition from enzymatically inactive trypsinogen to trypsin (Gertler et al., 1974; Bode & Huber, 1976; Bode et al., 1978; Perkins & Wüthrich, 1980).

Materials and Methods

Labeled BPTI was prepared with a modified version of the enzymatic replacement method of Sealock & Laskowski (1969), which was previously also used to replace Lys-15 by arginine, phenylalanine, and tryptophan (Jering & Tschesche, 1976). Bovine trypsin, bovine trypsinogen, and benzamidine were purchased from Merck A.G., and the synthetic dipeptide H-Ile-Val-OH was from Bachem A.G., Liestal. Anhydro-

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[1] Abbreviations used: BPTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor, Trasylol, Bayer Wuppertal, Germany); labeled BPTI, basic pancreatic trypsin inhibitor with the carbonyl carbon of the reactive site peptide bond Lys-15–Ala-16 enriched to 90% in ^{13}C, the peptide bond Arg-39–Ala-40 cleaved, and Arg-39 removed; NMR, nuclear magnetic resonance; ppm, parts per million; Me_4Si, tetramethylsilane.

0006-2960/80/0419-5711$01.00/0

Table I: ^{13}C Chemical Shifts in ppm from External Me_4Si for the Isotope-Enriched Carbon Atom of [1-^{13}C] Lys-15 in the Labeled Inhibitor in the Different Experiments of Figures 1-3 (T = 35 °C)

concn of labeled inhibitor (mM)[a]	protease (mM)[a]	benzamidine (mM)	$CaCl_2$ (mM)	H-Ile-Val-OH (mM)[a]	p^{2}H	chemical shift (±0.05 ppm)	see Figure
0.5					7.0	175.7	*b*
0.5		1.3	33		7.0	175.7	*b*
0.7	trypsin, 0.4	1.3	33		7.0	176.4, 175.7	1A
0.7	trypsin, 0.7	1.3	33		7.0	176.4	1B
0.7	trypsin, 0.7	1.3	33		2.0	175.7	1C
0.4	trypsinogen, 0.2	0.6	5		7.2	175.7	2A
0.4	trypsinogen, 0.5	0.6	5		6.4	175.7	2B
0.4	trypsinogen, 0.5	0.6	35		6.4	175.7	2C
0.4	trypsinogen, 0.5	0.6	35	0.6	6.4	176.4	2D
0.4	trypsinogen, 0.5	0.6	35	0.6	2.1	175.6	2E
0.7	anhydrotrypsin, 0.6	1.3	35		7.5	176.5	3A
0.7	anhydrotrypsin, 0.6	1.3	35		2.0	175.7	3B

[a] The concentrations of bovine trypsin and bovine trypsinogen were measured by using the absorption at 280 nm. The concentrations of labeled inhibitor, anhydrotrypsin, and H-Ile-Val-OH were calculated from the weight of the lyophilized material. [b] Richarz et al. (1979).

trypsin was prepared by Dr. C. A. Ryan according to the method of Ako et al. (1974) and was obtained as a gift from Drs. W. Bode and R. Huber at the Max Planck Institut für Biochemie in Martinsried.

The preparation of BPTI with the carbonyl position of Lys-15 enriched in ^{13}C and the characterization of this product by ^1H and ^{13}C NMR and chemical methods were described previously (Richarz et al., 1979). It was found that the reactive site peptide bond Lys-15–Ala-16 was re-formed in labeled BPTI. The covalent structure of labeled BPTI differs from that of native BPTI in that the peptide bond Arg-39–Ala-40 was cleaved and Arg-39 removed in the course of the enzymatic resynthesis. While the denaturation temperature at pH 6.5 was, by this modification, lowered from >95 °C in native BPTI to 85 °C, the solution conformation of native BPTI was found to be preserved in the labeled BPTI. Labeled BPTI had full inhibitory activity toward trypsin (Richarz et al., 1979). The inhibitory activity was identical before and after the experiments with trypsin, trypsinogen, and anhydrotrypsin.

The commercial trypsinogen was homogeneous in that it migrated as a single sharp band in sodium dodecyl sulfate–acrylamide gel electrophoresis (Weber et al., 1972; Price, 1976). The commercial trypsin gave a sharp band attributable to β-trypsin and several additional bands attributable to α-trypsin and inert proteins. Column chromatography by the method of Schroeder & Shaw (1968) confirmed these findings. Active site titration by the method of Chase & Shaw (1967) showed 80% trypsin activity. Freshly prepared trypsinogen solution showed no trypsin activity. Eighty percent trypsin activity was obtained after incubation of 2×10^{-3} M solutions of trypsinogen at 37 °C and pH 8.0 with 0.03 M $CaCl_2$ for 2–4 h (Kasche, 1976). Samples of trypsin for ^1H NMR experiments were obtained by incubation of 2×10^{-3} M ^2H_2O solutions of trypsinogen at 37 °C and p^{2}H 8.0 in the presence of 4×10^{-3} M benzamidine and 0.03 M $CaCl_2$ for 3 h. Comparison of the ^1H NMR spectra showed that the major component in the commercial trypsin was identical with the trypsin preparation thus obtained from trypsinogen.

The anhydrotrypsin was from the same source as the preparation used previously for the single-crystal X-ray studies of the anhydrotrypsin–BPTI complex (Huber et al., 1975). Activity tests performed for us by Dr. W. Bode at the Max Planck Institut für Biochemie in Martinsried showed less than 1% trypsin activity for the anhydrotrypsin both before and after the NMR experiments with the labeled BPTI.

For the NMR measurements, 0.0003–0.005 M protein solutions in ^2H_2O were used. The p^{2}H of the solutions was adjusted by the addition of minute amounts of ^2HCl or NaO^2H. The p^{2}H values are pH meter readings uncorrected for isotope effects (Kalinichenko, 1976; Bundi & Wüthrich, 1979). Fourier transform ^1H NMR spectra for the characterization and comparison of the different protein preparations were recorded at 360 MHz on a Bruker HX 360 spectrometer. Fourier transform ^{13}C NMR spectra were recorded at 25.16 MHz on a Varian XL 100 spectrometer. ^{13}C chemical shifts are quoted relative to external Me_4Si, where internal dioxane or benzamidine was used as the reference. The temperature in the probe was measured with a thermocouple, which was inserted directly into the samples. 40 000 to 80 000 transients were accumulated to obtain spectra like those in Figures 1–3. The free induction decays were multiplied with a decreasing exponential to improve the signal-to-noise ratio.

Results

Labeled BPTI was combined with trypsin, trypsinogen, or anhydrotrypsin under different solvent conditions to yield the corresponding enzyme complexes. Details of the individual experiments are summarized in Table I. The chemical shift of the ^{13}C-enriched carbonyl carbon of Lys-15 in the labeled BPTI was then measured in the solutions containing enzymes and the inhibitor.

Figure 1 shows the ^{13}C NMR spectra recorded in solutions of labeled BPTI and trypsin. Several features in Figure 1 are typical for all the experiments described here. Thus, the resonance of the ^{13}C-enriched carbonyl position of Lys-15 could readily be observed above the background of the natural abundance ^{13}C NMR spectrum of the proteins. Three sharp lines at 129.4, 130.8, and 136.0 ppm correspond to benzamidine. Benzamidine was added to all the solutions used for the NMR experiments (Mares-Guia & Shaw, 1965) to suppress self-digestion during the relatively long periods of time needed to record the ^{13}C spectra. It served also as an internal reference for relating protein chemical shifts to external Me_4Si. A small peak at 142.8 ppm, which is present in Figure 1A,C, and some of the spectra in Figures 2 and 3 is an instrumental artifact. In the experiment of Figure 1A, 0.4 mM trypsin was added to an 0.7 mM solution of labeled BPTI in neutral ^2H_2O, which further contained 1.5 mM benzamidine and 33 mM $CaCl_2$. Two separate resonance lines are seen at 176.4 and 175.7 ppm. More trypsin was then added to this solution up

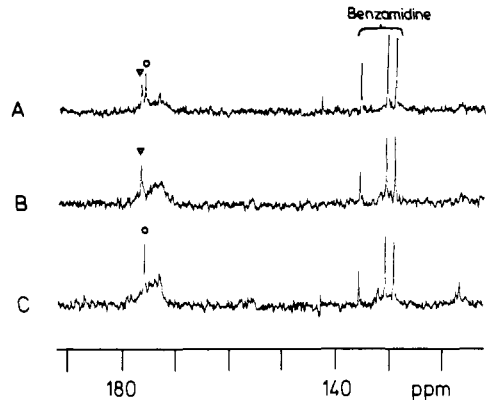

FIGURE 1: ^{13}C NMR spectra at 25.16 MHz of the complex between the labeled inhibitor and trypsin (solvent was ^2H_2O; T = 35 °C). The concentration of the labeled inhibitor was 0.7 mM. The solutions contained 1.3 mM benzamidine and 33 mM $CaCl_2$. Different trypsin concentrations and p^{2}H values were used in the individual experiments. (A) 0.4 mM trypsin, p^{2}H 7.0. Two resonances are observed at 175.7 ppm (○) and 176.4 ppm (▽). (B) 0.7 mM trypsin, p^{2}H 7.0. One resonance is observed at 176.4 ppm (▽). (C) 0.7 mM trypsin, p^{2}H 2.0. One resonance is observed at 175.7 ppm (○).

to a total enzyme concentration of 0.7 mM. The corresponding spectrum is shown in Figure 1B, where it is seen that the line at 175.7 ppm has disappeared and the line at 176.4 ppm has increased in intensity. Next, the p^{2}H in the solution of Figure 1B was changed to 2.0. As a consequence, the resonance of [1-^{13}C]Lys-15 in the labeled inhibitor was observed at 175.7 ppm (Figure 1C).

In Figure 1 the resonance line at 175.7 ppm can from comparison with the free labeled BPTI (Richarz et al., 1979) be assigned to unbound inhibitor. Since it is known that trypsin binds BPTI under the conditions of the experiments of Figure 1A,B (Tschesche, 1974) and labeled BPTI was shown to have the full inhibitory activity of BPTI, the line at 176.4 ppm must correspond to trypsin-bound labeled BPTI. Figure 1C then shows that the effect of the complexation reaction between trypsin and labeled BPTI on the ^{13}C chemical shift is fully reversible. This coincides with earlier observations that rapid lowering of the pH of a solution of trypsin–trypsin inhibitor complex from neutral to acidic pH yields predominantly virgin, i.e. reactive site intact, inhibitor (Hixson & Laskowski, 1970). The ^{13}C chemical shift of Figure 1C provides direct evidence that the reactive site peptide bond Lys-15–Ala-16 is intact after the complex dissociation. In reactive site cleaved labeled BPTI, the ^{13}C-enriched carboxyl resonance of Lys-15 would have an upfield protonation shift of ca. −3 ppm with a pK_a value of ~3.0 (Wüthrich, 1976; Richarz & Wüthrich, 1978).

The influence of trypsinogen on the ^{13}C chemical shift of labeled BPTI was investigated with the experiments of Figure 2. After the addition of 0.2 mM trypsinogen to a neutral solution of 0.4 mM labeled BPTI in ^2H_2O which contained also 0.6 mM benzamidine and 5 mM $CaCl_2$, only one resonance of the enriched carbon position was seen. The chemical shift was 175.7 ppm, as in free labeled BPTI (Figure 2A). No change of the chemical shift resulted after the trypsinogen concentration was increased to 0.4 mM (Figure 2B) and the $CaCl_2$ concentration was increased from 5 to 35 mM (Figure 2C). Next, 0.55 mM H-Ile-Val-OH was added to the solution of Figure 2C. As a consequence, the resonance of the enriched carbon was shifted to 176.4 ppm (Figure 2D). When the p^{2}H of the solution of Figure 2D was lowered to 2.1, the resonance was again observed at 175.6 ppm, which shows that the observed downfield shift was caused by reversible complex formation.

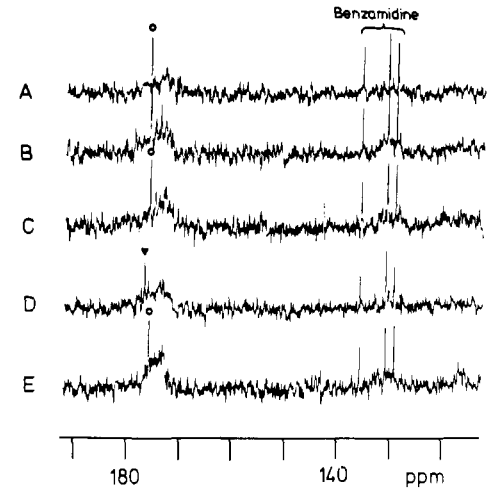

FIGURE 2: ^{13}C NMR spectra at 25.16 MHz of solutions containing the labeled inhibitor and trypsinogen (solvent was ^2H_2O; T = 35 °C). The concentration of the labeled inhibitor was 0.4 mM and the solutions contained 0.6 mM benzamidine. Other parameters were adjusted for the different experiments as described in the following. (A) 0.2 mM trypsinogen and 5 mM $CaCl_2$, p^{2}H 7.2. One resonance is observed at 175.7 ppm (○). (B) 0.5 mM trypsinogen and 5 mM $CaCl_2$, p^{2}H 6.4. One resonance is observed at 175.7 ppm (○). (C) 0.5 mM trypsinogen and 35 mM $CaCl_2$, p^{2}H 6.4. One resonance is observed at 175.7 ppm (○). (D) 0.5 mM trypsinogen, 35 mM $CaCl_2$, and 0.6 mM H-Ile-Val-OH, p^{2}H 6.4. One resonance is observed at 176.4 ppm (▽). (E) Same as (D), except that p^{2}H 2.1. One resonance is observed at 175.6 ppm (○).

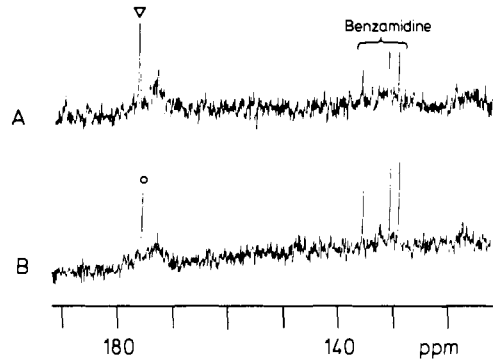

FIGURE 3: ^{13}C NMR spectra at 25.16 MHz of the complex between the labeled inhibitor and anhydrotrypsin (solvent was ^2H_2O; T = 35 °C). The solution contained 0.7 mM labeled inhibitor, 0.6 mM anhydrotrypsin, 1.3 mM benzamidine, and 35 mM $CaCl_2$. (A) p^{2}H 7.5. One resonance is observed at 176.5 ppm (▽). (B) p^{2}H 2.0. One resonance is observed at 175.7 ppm (○).

Figure 3 shows the results obtained when the labeled inhibitor was combined with anhydrotrypsin. It was previously shown by chemical methods and X-ray crystallography that BPTI forms a complex with anhydrotrypsin (Vincent et al., 1974; Ako et al., 1974; Huber et al., 1975). Figure 3A shows the spectrum of labeled BPTI bound to anhydrotrypsin. The resonance of the enriched carbon is observed at the same position as in the complex with trypsin. The chemical shift at p^{2}H 2.0 indicates again dissociation of the complex.

Discussion

Even though theoretical concepts for NMR chemical shifts are available (Abragam, 1962), structural interpretations are in practice almost entirely based on empirical comparison with related classes of molecules. For ^{13}C chemical shifts it is quite well established that they are largely affected by the hybridization of the carbon atom, the nature of the atoms bound to the carbon, and steric strain on the covalent bonds (Stothers, 1972; Wüthrich, 1976). Comparatively small effects are ex-

pected to arise from nonbonding interactions with the environment, such as the solvent. These qualitative ^{13}C shift–structure relations make labeled BPTI a suitable "probe" for studies of the influence of enzyme binding on the reactive site peptide bond of the inhibitor.

If a fully covalent tetrahedral adduct between trypsin and the carbonyl carbon of Lys-15 in BPTI were formed, which would correspond to the structure originally proposed on the basis of the X-ray data (Rühlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974), one would expect a large upfield chemical shift for the [1-^{13}C]Lys-15 resonance.[2] The observed 0.7-ppm downfield shift of the [1-^{13}C]Lys-15 resonance upon binding of labeled BPTI to trypsin (Table I) therefore shows that no covalent tetrahedral adduct is formed in the complex. This is in agreement with the conclusions from the interpretation of the refined X-ray data (Huber et al., 1974; Huber & Bode, 1978). Since identical data were obtained for the complexes of labeled BPTI with trypsin and anhydrotrypsin (Table I), it can further be concluded that the small downfield shift in the trypsin–BPTI complex does not manifest formation of an acyl–enzyme intermediate.

From the refined X-ray data it was further concluded that the carbonyl carbon of Lys-15 in the noncovalent trypsin–BPTI complex assumed a tetrahedrally distorted structure intermediate between sp^2 and sp^3 hybridization and that the γ-oxygen atom of Ser-195 from trypsin was at a distance of 2.6 Å from this carbonyl carbon (Huber et al., 1974; Huber & Bode, 1978). Since the differences between the X-ray structures of the Lys-15–Ala-16 peptide bond in free BPTI and the trypsin–BPTI complex are rather small ($\Delta\theta_4$ may be as small as 20°; R. Huber, private communication), it is difficult to assess what the corresponding ^{13}C chemical shift difference should be. As is discussed in more detail in the preceding paper (Baillargeon et al., 1980) on the basis of comparison with some model compounds, it appears that at present ^{13}C NMR studies of the type described in this and the preceding paper cannot be used to further characterize the state of the reactive site peptide bond in the noncovalent trypsin–inhibitor complex.

Similar results to those of Figure 1 were reported by Neves et al. (1979), Hunkapiller et al. (1979), and Baillargeon et al. (1980) for [1-^{13}C]Arg-63 and [1-^{13}C]Lys-63 soybean trypsin inhibitor bound to trypsin.[3] It thus appears that a downfield ^{13}C shift of ~0.8 ppm for the reactive site carbonyl position is a general phenomenon arising from the interactions of active serine proteases with their inhibitors. On this basis, some further qualitative conclusions on the structure of protease–inhibitor complexes can be drawn from the experiments with anhydrotrypsin and trypsinogen.

Since identical chemical shifts for [1-^{13}C]Lys-15 of labeled BPTI were observed in the complexes with trypsin and anhydrotrypsin (Table I), the ^{13}C NMR data would appear to indicate that direct interactions between the γ-hydroxyl of Ser-195 of trypsin and the reactive site peptide bond of the inhibitor do not play an important role in the interactions between the two proteins. From comparison of the X-ray structures of the BPTI complexes with trypsin and anhydrotrypsin, it was concluded that besides the conversion of Ser-195 to dehydroalanine there were no other significant structural differences (Huber et al., 1975). Overall it is then quite intriguing that identical chemical shifts were observed in the two complexes. From general experience with the solvent dependence of the ^{13}C chemical shifts of solvent-exposed carbonyl groups (Grathwohl et al., 1975; Wüthrich, 1976), removal of the γ-oxygen of Ser-195 of trypsin from its position in the BPTI–trypsin crystal structure would have been expected to cause a measurable variation of the chemical shifts of [1-^{13}C]Lys-15 of BPTI.[4]

Since the labeled inhibitor was shown to have an essentially identical average spatial structure as unmodified BPTI and was fully active toward trypsin (Richarz et al., 1979), it is to be expected, even though this was not experimentally verified, that its complexation with trypsinogen is similar to that of BPTI. It is then interesting that the ^{13}C NMR data appear to manifest an intermediate conformational state for trypsinogen in the complex with the inhibitor. From X-ray studies it had been concluded that in the trypsinogen–BPTI complex trypsinogen converts completely to an active trypsin-like molecular geometry (Bode et al., 1978). Apart from the different arrangement of the N termini in the trypsin and trypsinogen complexes, the only other significant structural difference between the two X-ray structures was enhanced disorder of the autolysis loop 142–152 in the trypsinogen complex. In the ternary complex trypsinogen–BPTI–H-Ile-Val-OH, the autolysis loop was found to be fixed as in the trypsin–BPTI complex (Bode et al., 1978). ^1H NMR studies (Perkins & Wüthrich, 1980) showed directly on the basis of solution evidence that trypsinogen in the complex with BPTI adopts a conformational state which is intermediate between the more flexible zymogen conformation and the more rigid conformation of the active enzyme. A trypsin-type conformation state was evidenced by the ^1H NMR data for trypsinogen in the ternary complex with BPTI and H-Ile-Val-OH (Perkins & Wüthrich, 1980). If one accepts that in the experiments of Figure 2A–C the labeled inhibitor was bound to trypsinogen, the ^{13}C NMR data (Table I) now indicate that the transition from a conformation-state intermediate between trypsinogen and trypsin in the trypsinogen–BPTI complex to a trypsin-type conformation in the ternary complex with H-Ile-Val-OH is transmitted across the protein–protein contacts between trypsinogen and BPTI. In a very general structural interpretation, one might conclude that the active protease exerts steric pressure on the reactive site peptide bond of BPTI which is not present in the complex with the inactive zymogen.

Acknowledgments

We thank S. Kupfer for skillful technical assistance, Drs. W. Bode, R. Huber, and C. A. Ryan for a gift of anhydrotrypsin, and Drs. M. Laskowski, Jr., and J. L. Markley for communicating their results prior to publication.

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[2] From observations in small organic molecules (e.g., Stothers, 1972), we estimate that the resonance of [1-^{13}C]Lys-15 should be ~50 ppm to higher field in a fully covalent tetrahedral adduct with trypsin than in free BPTI. One of the referees pointed out that this upfield shift should be smaller, i.e., 14 ppm.

[3] Hunkapiller et al. (1979) observed two resonances in solutions of the trypsin complex with [1-^{13}C]Arg-63 soybean trypsin inhibitor, which were 1.6 ppm apart. One of the lines was assigned to the acyl–enzyme complex. Neves et al. (1979) did not observe this second resonance line.

[4] One of the referees made the following comment. In anhydrotrypsin the γ-oxygen of Ser-195 is lost and the α- and β-carbons of Ser-195 become trigonal. As a consequence, there is a measurable conformational change of the β-carbon and there may be other subtle conformational changes which are below the significance level of the crystallographic analysis (Huber et al., 1975). All these will contribute to the chemical shift and may, by chance, compensate the effect of the loss of Ser O^{γ}.

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Structural Role of Phospholipids in Ubiquinol-Cytochrome *c* Reductase†

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ABSTRACT: The role of phospholipids in ubiquionol-cytochrome *c* reductase has been studied by the following methods: (1) removal and restoration of phospholipids, (2) circular dichroism measurements, and (3) phospholipase A_2 treatment. Over 90% of the phospholipids in the cytochrome b–c_1 III complex (a highly purified ubiquinol-cytochrome *c* reductase) can be removed by repeated precipitation with ammonium sulfate in the presence of 0.5% sodium cholate. The delipidated enzyme complex is inactive. Full restoration of enzymatic activity can only be achieved with a freshly prepared delipidated enzyme complex, made in the presence of 20% glycerol. As the age of the delipidated enzyme increased, the amount of activity restored decreased and the incubation time required to reach maximal activity increased. Removal of phospholipids from the cytochrome b–c_1 III complex resulted in an immediate decrease of ~15% in molar ellipticities in both the far-UV and the Soret regions. A further decrease in ellipticities was observed upon incubation of the delipidated enzyme at 0 °C in 50 mM phosphate buffer, pH 7.4. Replenishing phospholipids to the delipidated enzyme complex restored enzymatic activity and the molar ellipticity in both regions. The absolute requirement for phospholipids in the cytochrome b–c_1 III complex was also demonstrated by treatment of the enzyme with purified phospholipase A_2. The inactivation of the cytochrome b–c_1 III complex by phospholipase A_2 was not prevented by the presence of excess exogenous ubiquinone but was prevented by the presence of ethylenediaminetetracetic acid (EDTA). The enzymatic activity of the phospholipase A_2 treated complex is fully restorable upon the addition of EDTA and phospholipids.

The phospholipid (PL)[1] requirement in all four mitochondrial electron transfer complexes has been well documented (Ragan & Racker, 1973; McPhail & Cunningham, 1975; Hatefi et al., 1962; Baum et al., 1967; Yu et al., 1979a). The active complexes, as isolated, contain ~20% phospholipids. However, the degree of dependence of enzymatic activity on PL varies. Some regain activity when PL's are replaced by nonionic detergents (Yu et al., 1975), whereas others absolutely require

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[1] Abbreviations used: PL, phospholipid; Q, ubiquinone; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

0006-2960/80/0419-5715$01.00/0