Comparisons of Diisopropylfluorophosphate Derivatives of Chymotrypsin and Chymotrypsinogen by Phosphorus-31 Nuclear Magnetic Resonance

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SUMMARY

The nature of the differences in the active sites of α-chymotrypsin and chymotrypsinogen has been investigated by phosphorus-31 NMR studies of their diisopropylfluorophosphate derivatives. The phosphorus-31 resonance of the modified zymogen occurs 2 ppm upfield from that for the enzyme. An even greater separation is seen between diisopropylphosphoryl-neo-chymotrypsinogen and -α-chymotrypsin. A plausible interpretation of the chemical shift differences is based on the known structures for α-chymotrypsin, chymotrypsinogen and diisopropylphosphoryl-trypsin.

INTRODUCTION

The activation of chymotrypsinogen A to α-chymotrypsin is the most thoroughly studied conversion of an essentially inactive precursor protein to a fully active product. Consequently, this conversion serves as an especially important model system for understanding activation of the serine proteases by limited proteolysis. Two lines of experimental evidence suggest that the conformational changes which accompany the hydrolysis of peptide bonds in the activation of chymotrypsinogen are rather subtle. First, the atomic coordinates derived from X-ray diffraction studies of the crystalline proteins have revealed quite similar patterns of polypeptide chain folding. Even the arrangement of the catalytic residues at the active site were sufficiently similar that Freer et al. (1) were unable to infer with certainty which of the differences might be responsible for the much greater catalytic power of the enzyme. A second indication of similar active site structures was provided by the discovery that chymotrypsinogen can in fact catalyze the same reactions as chymotrypsin, albeit several orders of magnitude less effectively (2).

Our understanding of why chymotrypsin is a much better catalyst than chymotrypsinogen is far from complete. Certainly, refinements of electron density maps (3,4) will enhance our knowledge of the differences between the two proteins. However, solution studies are also essential. Some uncertainty will always exist in the atomic coordinates from crystallographic studies and some of the critical changes in conformation may well lie within these limits. It is also conceivable that lattice forces could induce some conformational changes in the exposed or flexible portions of the polypeptide chain in the crystalline state which might be reflected at the catalytic site.

Nuclear magnetic resonance spectroscopy is a technique capable of sensing small differences in conformation and providing information on the structure as well as motions of molecules in solution. We have taken advantage of the fact that both the enzyme (5) and the zymogen (6) react with diisopropylfluorophosphate to form a stable phosphoester bond with Ser-195 and have applied phosphorus-31 Fourier transform NMR[a] spectroscopy to probe the environment of the phosphorus nucleus in the two modified proteins.

MATERIALS AND METHODS

Both α-chymotrypsin and chymotrypsinogen A were purchased from Sigma (C-4129,CC-4879) and from Worthington (CDS,CGC). DIFP as obtained from Sigma was diluted with 2-propanol to a concentration of 1 M and stored over molecular sieves (Baker, activated Type 5A).

The proteins were dissolved in 0.05 M $CaCl_2$, 0.25 M KCl, 0.01 M Tris-Cl, pH 7.5 (buffer A). Chymotrypsinogen was routinely modified by adding DIFP in 2-propanol to a concentration of 25 mM and periodically adjusting the pH to 7.5 with 1 M NaOH. After 8 hr at room temperature the sample was dialyzed exhaustively at 4° against 0.25 M KCl, 0.01 M Tris-Cl, pH 7.5 (buffer B). In one instance, we modified chymotrypsinogen by 10 successive reactions with 1 mM DIFP. After each of the first 9 reactions (0.5 to 1 hr each at room temperature) the sample was dialyzed against buffer A for at least 2 hr. After the tenth reaction with DIFP, the sample was dialyzed exhaustively at 4° against buffer B. Chymotrypsin was modified by reaction at room temperature for 5 min at either 25 or 1 mM DIFP (both of which gave complete inactivation) and the samples dialyzed extensively at 4° against buffer B. Protein concentrations were determined spectrophotometrically using $A_{280}^{0.1\%} = 2.0$.

[a] Abbreviations: NMR, nuclear magnetic resonance; DIFP, diisopropylfluorophosphate; DIP-, diisopropylphosphoryl-; PRFT, partially relaxed Fourier transform; $^{31}P\{^{1}H\}$, proton noise decoupled phosphorus-31; TLCK, α-N-p-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethyl sulfonylfluoride.

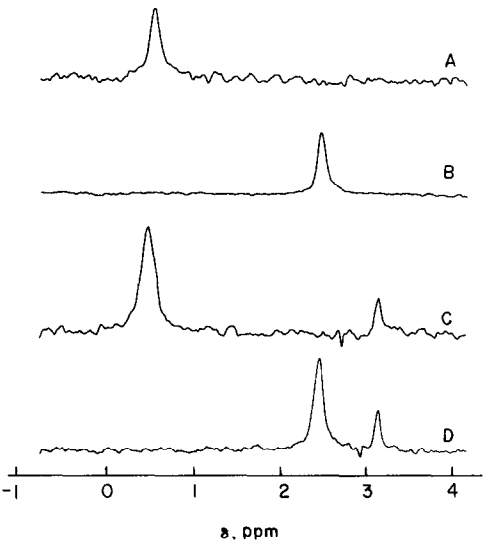

Fig. 1. Partially relaxed Fourier transform NMR spectra of the proton noise decoupled phosphorus-31 resonances of bovine DIP-α-chymotrypsin and DIP-chymotrypsinogens in 0.25 M KCl, 0.01 M Tris-Cl, pH 8.0 (at 25°C). A. DIP-α-chymotrypsin, 10.4 mg/ml, 32000 pulses; B. DIP-chymotrypsinogen, 54.4 mg/ml, 8000 pulses; C. trypsin-treated DIP-chymotrypsinogen, 10.7 mg/ml (total), 48000 pulses; and D. DIP-neo-chymotrypsinogen produced by the reaction of TLCK-treated α-chymotrypsin on DIP-chymotrypsinogen, 20.3 mg/ml, 16000 pulses.

The extent of modification of chymotrypsinogen was estimated by measuring the activity of the samples towards benzoyl tyrosine ethyl ester after incubation with bovine trypsin in buffer A and found to range from 54-62%. Reactions with trypsin were also used in two ways to determine whether or not the DIP group of the zymogen was attached to Ser-195. First, a ratio of zymogen to trypsin of 200:1 was utilized at room temperature and the reaction stopped after 5 min with corn trypsin inhibitor. This yielded 65% of the potential chymotrypsin activity. The second experiment used a ratio of 10,000:1 at 0° for 20 hr which gave 60% of the potential activity. DIP-neo-chymotrypsinogen was prepared by allowing TLCK-treated α-chymotrypsin to react for 1 hr with DIP-chymotrypsinogen in buffer A (10:1 zymogen:enzyme). After quenching the reaction with PMSF (20-fold molar excess) the sample was dialyzed overnight at 4°C against 800 volumes of buffer B.

PRFT $^{31}P\{^{1}H\}$ NMR spectra were acquired with a Varian XL-100-15 instrument equipped with an improved Nicolet 1080 FT system. Generally, the spectra were obtained over a 1000 Hz range using flip angles of about 60° (a 34.5 μsec pulse is equivalent to a 90° flip) and with a turn-around time of either 1.024 or 2.048 sec for 2K or 4K transient data points, respectively. The spectra were referenced to external 1 mM pyrophosphate[b], in D_2O, pD 10.4, contained in a concentric 5 mm capillary within the 12 mm sample tube. The external D_2O solvent in the capillary provided the ^{2}H lock. At this pD and ambient probe temperatures (37°) the single resonance of pyrophosphate occurs 7.36 ppm upfield from that of 6 mM H_3PO_4 in water. As it is customary for

[b]Pyrophosphate is apparently stable for months at pD 10-11 and room temperatures.

^{31}P NMR, the chemical shifts of the DIP derivatives are reported relative to external H_3PO_4 using the above conversion. The spectrometer frequency for ^{31}P was 40.5 MHz (^1H, 100 MHz).

RESULTS

Figs. 1A and 1B show the $^{31}P\{^1H\}$ PRFT NMR spectra of DIP-α-chymotrypsin and DIP-chymotrypsinogen, respectively. The chemical shift of the sole phosphorus resonance of DIP-α-chymotrypsin (0.52 ppm) occurs considerably downfield from that of triisopropylphosphate (3.2 or 5.9 ppm) but is not so different from those of other triesters [tri-n-butylphosphate, -1.0 ppm (7)]. (Hydrolyzed but otherwise unreacted DIFP shows ^{31}P resonances at 0.85 and -5.5 ppm in buffer B, pH 7.6, which correspond to the di- and moniosopropyl derivatives, respectively). There is, however, a substantial difference in the chemical shift for DIP-α-chymotrypsin and that for DIP-chymotrypsinogen (2.46 ppm) indicating a more shielded phosphorus nucleus in the modified zymogen.

The ^{31}P chemical shifts of DIP-α-chymotrypsin were slightly concentration dependent and moved downfield by 6-7 Hz between 11 and 2 mg/ml while those for DIP-chymotrypsinogen showed little, if any, significant change with concentration. In addition, the ^{31}P line widths for the modified enzyme were generally two to three times as broad as those for the modified zymogen (∿3 Hz). Both of these observations are consistent with a greater concentration dependent self-association for DIP-α-chymotrypsin than for chymotrypsinogen.

During inactivation with DIFP we consistently observed precipitation of protein at 25 mM reactant which may have resulted from denaturation of the protein by isopropanol. To exclude the possibility that isopropanol irreversibly alters the conformation of the chymotrypsinogen which remained in solution, we prepared DIP-chymotrypsinogen by 10 successive reactions at 1 mM DIFP, in which the isopropanol concentration never exceeded 0.1% (see Methods). No precipitation was observed in these reactions and the ^{31}P chemical shift of the resulting DIP-chymotrypsinogen was 2.44 ppm. We

conclude therefore that the presence of 2% isopropanol during the reaction does not irreversibly alter the conformation of chymotrypsinogen at least as reflected by the ^{31}P chemical shifts of the DIP moiety attached at the active site.

To verify that the DIP group on chymotrypsinogen was attached to Ser-195 we treated DIP-chymotrypsinogen with trypsin to partially convert it to DIP-chymotrypsin. Fig. 1C is the $^{31}P\{^{1}H\}$ spectra of the fast activation sample (slow activation gave the same results). The downfield peak in this spectrum occurs at nearly the same chemical shift (0.48 ppm) as DIP-α-chymotrypsin (Fig. 1A) which demonstrates that Ser-195 is the site of attachment of DIP in the zymogen.

The chemical shift of the second peak (3.16 ppm) in Fig. 1C, however, does not correspond to that for DIP-chymotrypsinogen. Instead, it agrees nicely with the chemical shift for chymotrypsin-treated DIP-chymotrypsinogen, i.e., one of the DIP-neo-chymotrypsinogens (8)(Fig. 1D). DIP-neo-chymotrypsinogen was presumably generated during preparation of the sample for Fig. 1C from proteolytic attack on the zymogen by the unmodified chymotrypsin which had arisen from the tryptic-activation of the unmodified chymotrypsinogen.

DISCUSSION

Limited proteolysis is now recognized as a biological control mechanism of wide-spread importance (9). A prominent class of proteins that are activated by limited proteolysis are precursors of serine proteases. Included among these are chymotrypsinogen and trypsinogen, procoonase, blood clotting factors, plasminogen, some elements of the complement system, proacrosin, and prekallikrien. The study of the activation of chymotrypsinogen is therefore directly relevant to understanding the activation of many different zymogens involved in a wide diversity of biological control systems.

Earlier ^{1}H NMR investigations of α-chymotrypsin and chymotrypsinogen A did not detect any differences between the pH dependence of the chemical

shifts for the Asp-102 and His-57 hydrogen bonded protons of the two proteins except for a slight extra perturbation in the enzyme curve near pH 5.5 (10,11). More recently, however, Markley and coworkers (12) have followed the behavior of the C-2 proton of His-57 with pH and found a considerably elevated pK for the zymogen. In addition, the difference in pK between the two proteins appears to coincide with those obtained from the pH dependence of deacylation of p-guanidinobenzoyl-chymotrypsin and -chymotrypsinogen derivatives (2). Our ^{31}P NMR studies of DIP-α-chymotrypsin, DIP-chymotrypsinogen, and DIP-neo-chymotrypsinogen seem more in line with Markeley's His-57 titration work. A chemical shift of 2 ppm between the phosphorus atoms in the modified enzyme and zymogen is substantial, and indicates that the ^{31}P NMR of these DIP derivatives is a sensitive probe of the active site region.

One structural factor capable of producing sizable chemical shifts is hydrogen bonding. The X-ray crystallographic work of Stroud et al. (13) on DIP-trypsin places the phosphorus atom very near the position determined for the acyl carbon of the proposed tetrahedral intermediate (14) of a normal substrate. The close structural analogy between DIP-trypsin (13) and α-chymotrypsin (1) suggests that a similar position may obtain in DIP-α-chymotrypsin as well. Consequently, it is reasonable to expect the N-H groups of Ser-195 and Gly-193 to form hydrogen bonds with the tetrahedral phosphoryl oxygen of DIP-chymotrypsin as it does in DIP-trypsin. Wright (3,4) noted, however, that the N-H of Gly-193 in chymotrypsinogen would not be able to form such a hydrogen bond due to rotation of this chain segment relative to that in chymotrypsin.

It seems unlikely that the absence of a single hydrogen bond is sufficient to account for all the chemical shift difference we have found between DIP-α-chymotrypsin and DIP-chymotrypsinogen since hydrogen bonding to the phosphoryl oxygen of several diesters produced average downfield shifts of only 0.75 ppm (15). Consequently, additional factors, such as interactions with solvent or geometric differences, need to be considered.

Although there are differences in tightly bound water between chymotrypsin and chymotrypsinogen (1) similar information is not presently available for the DIP derivatives and the possible role of solvent is difficult to assess. On the other hand, Gorenstein (16) has suggested that the chemical shifts of phosphoesters are quite sensitive to changes in the geometry of these compounds and are related to the smallest bond angle at the phosphorus atom. Consequently, the remaining chemical shift differences could result from a change in P-O bond angle. Although there could well be other differences in chymotrypsin and chymotrypsinogen structures, such as changes in polarization of the Ser-195 γ-oxygen atom, which could contribute to the positions of the ^{31}p resonances, available data do not allow their effects to be evaluated quantitatively.

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