NMR Structural Studies of the Tight Complex between a Trifluoromethyl Ketone Inhibitor and the 85-kDa Human Phospholipase A_2

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ABSTRACT: Arachidonyl trifluoromethyl ketone ($AACOCF_3$) is a slow- and tight-binding inhibitor of the human cytosolic phospholipase A_2 ($cPLA_2$) [Street et al. (1993) *Biochemistry 32,* 5935]. ^{19}F and ^{13}C NMR experiments have been carried out to elucidate the structure of the $cPLA_2$·$AACOCF_3$ complex. One mole of $AACOCF_3$ per mole of enzyme is tightly bound in the active site while excess molar equivalents of the inhibitor associate loosely and nonspecifically with hydrophobic regions of the protein. Incubation of the $cPLA_2$·$AACOCF_3$ complex with a 10-fold molar excess of a structurally related inhibitor allows the slow dissociation of the enzyme–inhibitor complex to be followed with ^{19}F NMR. These results establish that the bound inhibitor is in slow exchange with the free ligand and that inhibition of the $cPLA_2$ by $AACOCF_3$ is not due to irreversible modification of the protein. $AACOCF_3$ labeled with ^{13}C at the carbonyl position was used to determine the nature of the bound inhibitor species. A comparison of the ^{13}C NMR chemical shift value obtained from labeled enzyme–inhibitor complex (δ_C 101.0 ppm) with the chemical shift values obtained from model compounds suggests that the enzyme-bound inhibitor species is a charged hemiketal. These results are very similar to those obtained previously with α-chymotrypsin and a peptidyl trifluoromethyl ketone inhibitor [Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26,* 7603] and, by analogy with the serine proteases, a structural model for the $cPLA_2$·$AACOCF_3$ complex is proposed.

Phospholipases A_2 (PLA_2)[1] (EC 3.1.1.4) catalyze the hydrolysis of the ester linkage at the *sn*-2 position of phospholipids to produce a free fatty acid and a lysophospholipid (van Deenan & de Haas, 1963). Most of the PLA_2s purified and characterized so far are from the family of low molecular weight secreted enzymes ($sPLA_2$) (Verheij et al., 1981; Dennis, 1983; Seilhamer et al., 1989; Kramer et al., 1989). These enzymes may be isolated from a variety of different sources, such as venoms, pancreatic secretions, human synovial fluid, plasma, and platelets.

Recently, a new type of PLA_2 found in the cytosol of cells ($cPLA_2$) has been characterized. The $cPLA_2$ has been purified from various sources such as platelets (Kramer et al., 1987; Kim et al., 1991), macrophages (Leslie et al., 1987; Wijkander & Sundler, 1989), spleen (Wijkander & Sundler, 1991), kidney (Gronich et al., 1990), and the human monocytic cell line U937 (Clark et al., 1990, 1991; Diez & Mong, 1990; Kramer et al., 1991). The $cPLA_2$ has a high molecular mass (85 kDa), is activated by submicromolar concentrations of calcium (Clark et al., 1991; Kramer et al., 1991; Tremblay et al., 1992), and shows some selectivity toward the hydrolysis of arachidonyl-containing phospholipids (Diez & Mong, 1990; Clark et al., 1991; Kramer et al., 1991; Hanel et al., 1993). In the cellular environment the activity of the $cPLA_2$ is controlled by a number of signal transduction pathways (Lin et al., 1992; Kramer et al., 1993; Nemenoff et al., 1993; Xing & Mattera, 1992). The observation of these mechanisms for the cellular regulation of the $cPLA_2$, along with the other favorable properties of this enzyme, has led many to propose that it might play an important role in regulating the biosynthesis of leukotrienes and prostaglandins.

The available evidence suggests that there are very few mechanistic similarities between $sPLA_2$ and the $cPLA_2$. Within the primary amino acid sequence of the $cPLA_2$ there are no regions which share homology with the active site sequences of $sPLA_2$. Thus it is likely that there are significant differences in the architectures of the catalytic sites. Furthermore, although both types of PLA_2 require calcium for phospholipase activity, the function of this cofactor is different for each. For $sPLA_2$, the calcium ion plays a central role in the catalytic mechanism by initially polarizing the susceptible ester group of the substrate and subsequently by stabilizing the transition state of the reaction (Scott et al., 1991). In contrast, the evidence suggests that for the $cPLA_2$ the calcium ion does not participate directly in the chemical mechanism of catalysis, but that it functions in promoting interaction of the $cPLA_2$ with the lipid–water interface (Wijkander & Sundler, 1992; Ghomashchi et al., 1992).

To further highlight the differences between $sPLA_2$ and the $cPLA_2$, a number of potent substrate/transition-state analog inhibitors are available for $sPLA_2$ (Yuan et al., 1987; Jain et al., 1989, 1991; Ransac et al., 1990; Yu et al., 1990), most of which show little if any inhibitory activity against the $cPLA_2$ (I. P. Street and M. H. Gelb, unpublished results). Moreover, selective inhibitors of the $cPLA_2$ versus $sPLA_2$ have also recently been described (Street et al., 1993). Electrophilic ketone analogs of arachidonic acid are a novel class of slow- and tight-binding inhibitors of the $cPLA_2$. For the most potent compound in this series, arachidonyl trifluoromethyl ketone ($AACOCF_3$), the equilibrium dissociation constant of the $cPLA_2$–inhibitor complex in the presence of calcium and a lipid–water interface is less than 5×10^{-5} mole fraction. In comparison, the equilibrium dissociation constant for the $AACOCF_3$·$sPLA_2$ complex is greater by over 4 orders of

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[1] Abbreviations: PLA_2, phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; $cPLA_2$, 85-kDa cytosolic phospholipase A_2; $AACOCF_3$, $AACOCF_2Cl$, analogs of arachidonic acid having the hydroxyl group replaced by a CF_3 or CF_2Cl group, respectively; AA^*COCF_3, analog of $AACOCF_3$ labeled with ^{13}C at the carbonyl position; FID, free induction decay; BSA, bovine serum albumen.

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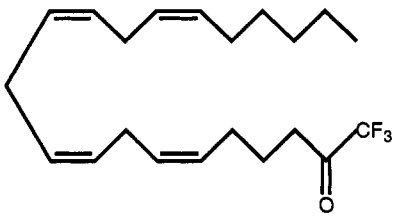
magnitude (0.1 mole fraction). These observations all suggest that there are fundamental differences in the catalytic mechanisms and active-site structures of the $cPLA_2$ and $sPLA_2$.



$AACOCF_3$

The use of stable transition-state analog/inhibitors in conjunction with NMR spectroscopy has often provided useful insights into the catalytic mechanism of an enzyme (Bégué & Bonnet-Delpon, 1991). Structural studies using NMR have been performed on $sPLA_2$s (Fanni et al., 1989; Fisher et al., 1989; Peters et al., 1992; Dupureur et al., 1992) and on $sPLA_2$–inhibitor complexes (Plesniak et al., 1993; Williamson et al., 1989). It is well known that ^{19}F NMR is suited to demonstrating ligand- or substrate-binding events. The inherent sensitivity of the ^{19}F nucleus together with the well-known line-broadening effect of high molecular weight complex formation gives this approach its utility. However, ^{19}F NMR chemical shifts are not a useful reporter of the nature of the bound inhibitor because a single species can have a range of chemical shifts that is dependent on the polarity of its environment. Additional information on the bound ligand can often be obtained from ^{13}C NMR measurements. The chemical shift values of this nucleus are relatively insensitive to the polarity of their environment, and consequently information can be gained on the nature of the enzyme-bound inhibitor from chemical shift measurements made on simple model compounds. For example, work by Abeles and colleagues on the NMR of α-fluoroketo peptides bound to α-chymotrypsin (Liang & Abeles, 1987; Parisi & Abeles, 1992) led to structural conclusions that were later verified when the X-ray structure of the enzyme–inhibitor complex was solved (Brady et al., 1990). ^1H, ^{13}C, and ^{19}F NMR experiments established the bound inhibitor as an ionized hemiketal formed with a serine group in the active site of the enzyme. This conclusion was largely based on ^{13}C NMR data of the enzyme–inhibitor complex. Model compounds were then used to establish the chemical shifts of the prospective species. The study also confirmed that ^{13}C chemical shifts are relatively insensitive to the milieu of the molecule. Thus, it is possible to make conclusions on the structure of the inhibitor bound to the enzyme based on such model studies.

In this communication we describe a series of experiments in which we have used ^{19}F and ^{13}C NMR spectroscopy to elucidate the structure of the $AACOCF_3$·$cPLA_2$ complex. Comparison of these results with those obtained in a previous study of α-chymotrypsin and a trifluoromethyl ketone inhibitor (Liang & Abeles 1987), suggests that the structure and environment of the trifluoromethyl ketone group in the enzyme–inhibitor complexes of $cPLA_2$ and α-chymotrypsin are very similar. By analogy with serine proteases, our data support a model that, on binding in the active site of the $cPLA_2$, $AACOCF_3$ forms a hemiketal with a serine or threonine group and that the pK_a of the bound hemiketal is substantially lower than that of the simple methyl hemiketal of $AACOCF_3$ formed in free solution. These results suggest that there may be significant mechanistic similarities between the $cPLA_2$, serine proteases, and other lipases in which the reaction mechanism proceeds through formation of an acyl–enzyme intermediate.

MATERIALS AND METHODS

$cPLA_2$ Preparation. The $cPLA_2$ was prepared according to the method of Street et al. (1993). The large amounts of protein required for the NMR experiments were purified using a 10 × 10 cm column of Fractogel EMD DEAE-650 (M) (E. Merck), and a 24 × 5 cm column of Phenyl Sepharose High Performance (Pharmacia LKB). The purified $cPLA_2$ was then concentrated to a volume of 1–2 mL with a Centriprep-30 concentrator and dialyzed overnight against 100 mL of a buffer containing 50 mM Tris-HCl (pH 8.1), 1 mM EDTA, 200 mM KCl, and 20% (v/v) D_2O. The dialyzed protein was then concentrated further with a Centricon-30 to a final volume of 0.35 mL. The final enzyme concentration was determined from the absorbance at 280 nm (E_{280} 0.827 mg^{-1} mL^{-1} cm^{-1}).

Arachidonyl Trifluoromethyl Ketone-2-[^{13}C](AA$COCF_3$).* AA^*COCF_3 was prepared in a manner similar to that used before for the nonlabeled material (Street et al., 1993; Boivin et al., 1992). To a solution of sodium trifluoro[1-^{13}C]acetate (50 mg, 0.365 mmol) in THF was added mesyl chloride (28 μL, 0.365 mmol). After 30 min at room temperature this solution was added to the acid chloride of arachidonic acid (30 mg, 0.098 mmol) in THF, followed by pyridine (78 μL, 0.784 mmol). After 5 h the product was worked up as described previously.

NMR Spectra. Solutions of $cPLA_2$ (0.35 mL) were typically 0.2–0.7 mM for ^{19}F, and 0.7 mM for ^{13}C NMR studies. Stock solutions of $AACOCF_3$ and AA^*COCF_3 were stored in toluene under argon at −20 °C. Prior to the experiments the appropriate amount of the toluene solution was dried under a stream of nitrogen gas and then the inhibitor was redissolved in DMSO. Additions of the inhibitor to the buffered enzyme solution were made directly from the DMSO stock (typically 2–3 μL aliquots).

^{19}F NMR spectra were recorded at 282.4 MHz on a Bruker AMX 300 spectrometer using a 90° tip angle and 0.1-s repetition rate. The probe head temperature was 300 K, and the signal was collected in 30 min. The FIDs were processed using Felix (Hare Research). In order to reduce the intense, broad signal arising from fluorine-containing materials within the probe head, the spectrometer dead time was set to the dwell time of the acquisition. The time-domain data were then right-shifted by one complex point and the first 10 complex points recalculated using a linear back prediction algorithm. A 25-Hz line-broadening preceded FT. Chemical shifts were referenced to internal trifluoroacetic acid (δ = 0.0).

^{13}C NMR spectra were recorded at 125.76 MHz on a Bruker AMX 500 spectrometer. A 30° tip angle and 1.3-s repetition rate was used to collect the signal over a 24–64-h period. Spectra were referenced to DMSO (δ = 39.5), and, unless otherwise stated, a 25-Hz line-broadening factor was applied prior to FT. The probe temperature was 295 K, and spectra were recorded with composite pulse decoupling of ^1H.

RESULTS

Stoichiometry and Reversibility of the $AACOCF_3$·$cPLA_2$ Complex. ^{19}F NMR spectroscopy was used to establish the stoichiometry of the $AACOCF_3$·$cPLA_2$ complex and whether its formation was reversible. A spectrum of $AACOCF_3$ in aqueous solution at concentration of 0.15 mM showed a peak at −4.9 ppm. Since $AACOCF_3$ is only sparingly soluble in aqueous buffers, this signal probably arises from an aggregated form of the inhibitor. During the first 30-min aquisition period after the addition of a single mole equivalent of $AACOCF_3$ (0.67 mM) to $cPLA_2$ (0.7 mM), the sharp signal at −4.9 ppm

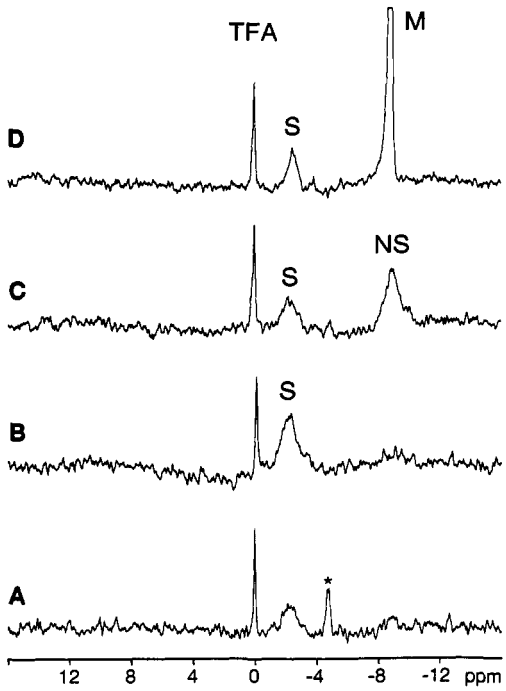


FIGURE 1: ^{19}F NMR spectra (282.4 MHz) of $AACOCF_3$ and $cPLA_2$ in aqueous buffer solution. (A) Equimolar concentrations of enzyme (0.7 mM) and $AACOCF_3$ (0.67 mM) during the first 30-min aquisition period after mixing the enzyme and inhibitor. An asterisk marks the peak from aggregated $AACOCF_3$ which has not yet bound to the enzyme active site. (B) Equimolar concentrations of enzyme (0.7 mM) and $AACOCF_3$ (0.67 mM) after binding of the inhibitor is complete (~60 min after mixing). The broad signal at −2.2 ppm labeled "S" corresponds to specifically bound inhibitor. (C) The spectrum from 0.35 mM $cPLA_2$ and 1.4 mM $AACOCF_3$. The nonspecifically bound $AACOCF_3$ is labeled "NS". (D) The spectrum from 0.27 mM $cPLA_2$, 1.0 mM $AACOCF_3$, and 41 mM Triton X-100. The signal for inhibitor dispersed in the micelles is labeled "M". All samples contained TFA as an internal chemical shift reference.

slowly reduced in intensity and a broad signal at −2.2 ppm appeared (Figure 1A). The broad signal had a half-width of 220 ± 20 Hz. After approximately 60 min, only the signal at −2.2 ppm was observed (Figure 1B). Addition of a second molar equivalent of $AACOCF_3$ had no effect on the −2.2 ppm signal intensity, but a second, new broad signal appeared at −8.8 ppm ($\Delta\nu_{1/2}$ = 245 ± 20 Hz). The large half-width of the signal at −8.8 ppm suggested that this species was also associated with the protein. Further additions of $AACOCF_3$ to the sample increased the area of the signal at −8.8 ppm only (Figure 1C). It was postulated that the lower-field broad signal arose from $AACOCF_3$ bound in the active site of the $cPLA_2$ and that the higher-field signal might be attributable to inhibitor associated in a nonspecific manner with enzyme. Previously it has been shown that in the absence of a lipid–water interface $sPLA_2$ can form aggregates with monomeric substrates and inhibitors (Bukowski & Teller, 1987; Yuan et al., 1990; Rogers et al., 1992). This phenomenon is observed in an aqueous environment because hydrophobic compounds associate relatively nonspecifically with hydrophobic areas of the protein. Such aggregate formation between the $cPLA_2$ and the hydrophobic $AACOCF_3$ in the aqueous buffer system used in these experiments could account for the large half-width of the signal at −8.8 ppm. Furthermore, the fact that these weakly associated species likely see a range of chemical environments causes the signals to resonate over a limited chemical shift range. This then causes the apparent linewidth to be larger than anticipated. The relatively weak nature of the association between the $cPLA_2$ and the excess molar equivalents of $AACOCF_3$ was demonstrated by addition of Triton X-100 detergent to the solution (Figure 1D). This

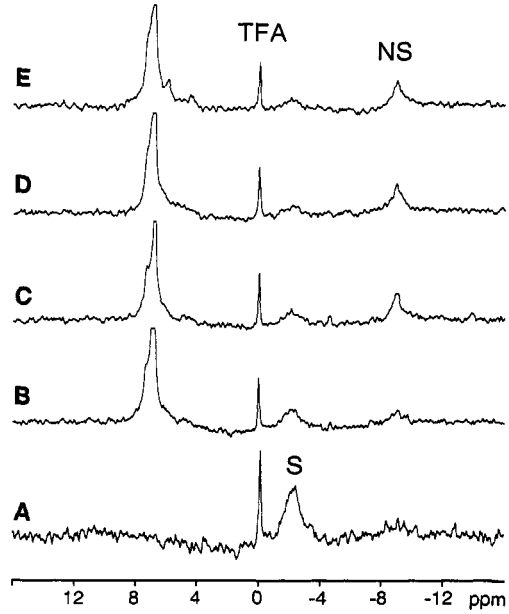


FIGURE 2: ^{19}F NMR spectra (282.4 MHz) of a $cPLA_2$ competition experiment between $AACOCF_3$ and $AACOCF_2Cl$. (A) The spectrum of equimolar concentrations of enzyme (0.7 mM) and AA^*COCF_3 (0.67 mM). The spectra of the sample shown in panel A at the following times after addition of 7.6 mM $AACOCF_2Cl$: (B) 1 h, (C) 24 h, (D) 48 h, and (E) 72 h.

caused a slight decrease in the half-width of the signal at −2.2 ppm ($\Delta\nu_{1/2}$ = 200 ± 20 Hz) and the resonance at −8.8 ppm to sharpen dramatically ($\Delta\nu_{1/2}$ = 25 ± 5 Hz). This suggests that the presence of the detergent can disrupt the nonspecific association of the enzyme and the inhibitor. However, the presence of the detergent had little effect on the resonance from the $AACOCF_3$ bound in the active site of the $cPLA_2$. Broadening of the bound ligand signal due to chemical exchange is very unlikely given the slow dissociation of the $cPLA_2$·$AACOCF_3$ complex (see competition experiment below).

Street et al. (1993) have demonstrated that in the absence of free calcium the $AACOCF_3$·$cPLA_2$ complex slowly dissociates to produce free substrate and active enzyme. The reversibility of the $AACOCF_3$·$cPLA_2$ complex was demonstrated here by a competition experiment between AA^*COCF_3 and a large excess of $AACOCF_2Cl$. $AACOCF_2Cl$ is also a slow-binding inhibitor of the $cPLA_2$ but is less potent than $AACOCF_3$ (Street et al., 1993). Again ^{19}F NMR was used to report the process, and the ^{13}C labeled inhibitor was used so that the competition could be followed by both ^{19}F and ^{13}C NMR. The $cPLA_2$·AA^*COCF_3 complex was allowed to form as before, and then an 11-fold molar excess of $AACOCF_2Cl$ was added. The ^{19}F NMR spectra corresponding to this experiment are shown in Figure 2. Over 72 h, the signal at −2.2 ppm progressively decreased in intensity with a concomitant increase in the intensity of a signal at −8.8 ppm. The peaks downfield of the reference standard were ascribed to the added $AACOCF_2Cl$ inhibitor and the $AACOCF_2Cl$·$cPLA_2$ complex. The excess of $AACOCF_2Cl$ and broad, overlapping signals made assignment of low-field peaks impossible. These results are consistent with the slow dissociation of the $AACOCF_3$ complex to produce the free enzyme and free $AACOCF_3$, which then must compete for the $cPLA_2$ binding site with the large excess of $AACOCF_2Cl$. In the absence of competing ligand, the signal from the $AACOCF_3$·$cPLA_2$ complex remained unchanged for longer than 48 h. Therefore, it is likely that the slow disappearance of the resonance from the $AACOCF_3$·$cPLA_2$ complex observed in the presence of $AACOCF_2Cl$ is due to the exchange of the

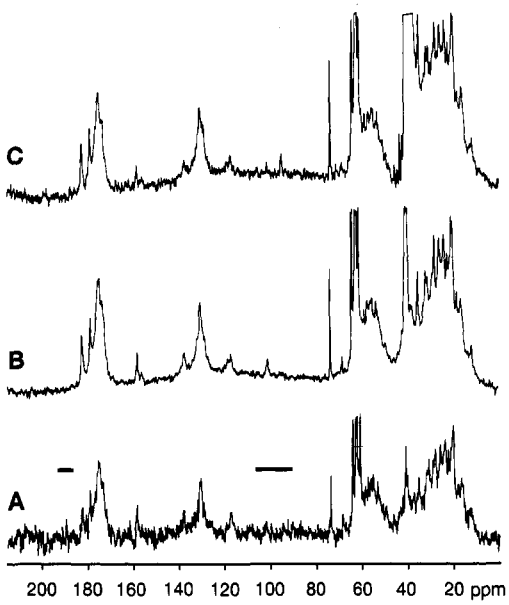


FIGURE 3: ^{13}C NMR spectra (125.76 MHz) of $cPLA_2$ with and without inhibitors. (A) Spectrum of $cPLA_2$ in aqueous buffer solution (5000 scans). Horizontal bars indicate frequency ranges where model studies showed that inhibitor signals might resonate. (B) Spectrum (178 980 scans) of $cPLA_2$ (0.7 mM) with 1 equiv of added AA*$COCF_3$ (0.67 mM). (C) Spectrum (79 184 scans) obtained 72 h after addition of 7.6 mM of $AACOCF_2Cl$ to the sample shown in panel B. The peak at 39.5 ppm in panels B and C is due to the added DMSO.

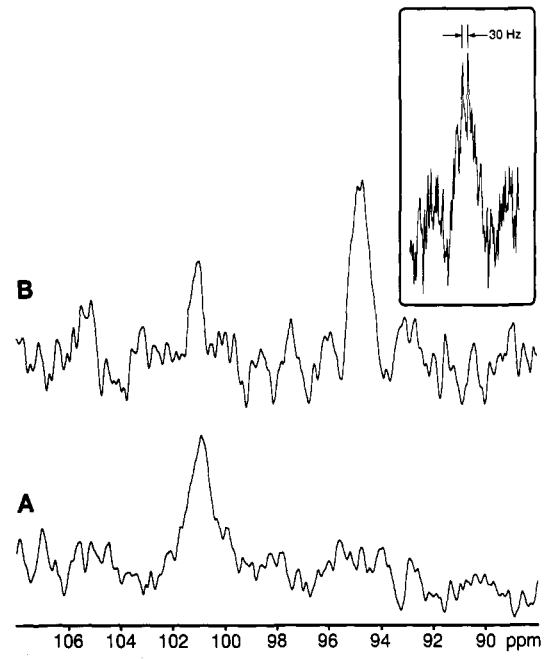


FIGURE 4: ^{13}C NMR spectra (125.76 MHz) of $cPLA_2$ with and without inhibitors: expansion of the 88–108 ppm spectral region. (A) Spectrum of $cPLA_2$ (0.7 mM) with 1 equiv of added AA*$COCF_3$ (0.67 mM). (B) 72 h after addition of 7.6 mM of $AACOCF_2Cl$ to the sample shown in panel A. (Inset) The higher-field signal from panel B with reduced line broadening used prior to FT. The heteronuclear coupling constant is apparent.

ligands bound to the protein and not due to denaturation of the enzyme and release of the bound $AACOCF_3$.

^{13}C NMR of the $AACOCF_3$·$cPLA_2$ Complex. ^{13}C NMR and the appropriately labeled inhibitor can be used to report the possible formation of a covalent complex, the type of amino acid side chains that might be involved, and whether the complex is charged or neutral (Liang & Abeles, 1987; Bégué & Bonnet-Delpon, 1991). $AACOCF_3$ enriched with ^{13}C at the carbonyl position (AA*$COCF_3$) was prepared and mixed with $cPLA_2$ in a 1:1 molar ratio. The ^{13}C NMR spectrum of the enzyme–inhibitor complex showed a broad signal at 101.0 ppm (see Figure 3B and Figure 4A). The large apparent line width of the signal from the enzyme–inhibitor complex ($\Delta\nu_{1/2}$ = 140 ± 10 Hz) obscured the expected heteronuclear coupling constant ($^2J_{CF}$ = 30 Hz). This signal was not observed in the spectrum of the enzyme alone (Figure 3A). The resonance due to the nonspecifically bound inhibitor was obtained by competing the AA*$COCF_3$ from the active site of the $cPLA_2$ with a 11-fold molar excess of $AACOCF_2Cl$, as described for the ^{19}F NMR experiments. After 72 h the signal at 101.0 ppm had significantly decreased in area, and a new, sharper signal at 94.5 ppm was evident (Figure 3C and Figure 4B). Under signal processing conditions where a smaller (5 Hz) line-broadening was applied, the quartet structure due to the heteronuclear coupling of the carbonyl carbon and the adjacent trifluoromethyl group was visible (inset to Figure 4B). We again postulated that the signal at 101.0 ppm was due to AA*$COCF_3$ bound in the active site of the $cPLA_2$, and the sharper signal at 94.5 ppm arose from AA*$COCF_3$ nonspecifically bound to $cPLA_2$.

^{13}C NMR of Model $AACOCF_3$ Complexes. Spectra of AA*$COCF_3$ in CD_3OD/D_2O solution showed a quartet ($^2J_{CF}$ = 30 Hz) centered at 94.5 ppm and a second quartet at 97.4 ppm. These were assigned to, respectively, the hydrate and the methyl hemiketal of $AACOCF_3$. Sequential additions of 0.1 M NaOD to the solution caused the signals to progressively move downfield. A limiting value for the chemical shift of the monoanion of the hydrate was established as 97.2 ppm and as 101.0 ppm for the anion of the methyl hemiketal. The chemical shift values of hydrated $AACOCF_3$ were also recorded at pH 8.0 in mixed micelles with Triton X-100 (94.5 ppm) and with a 1:1 molar ratio of BSA (94.5 ppm). A comparison of the results obtained from the model compounds with the chemical shift of the $AACOCF_3$·$cPLA_2$ complex (101.0 ppm) suggests that an ionized hemiketal is formed upon binding $AACOCF_3$ in the active site of the $cPLA_2$. The chemical shift of the nonspecifically bound $AACOCF_3$ (94.5 ppm) is consistent with this species being the neutral hydrate of $AACOCF_3$.

It is also possible that other types of nucleophile might form a complex with $AACOCF_3$ in the active site of the $cPLA_2$. It has been reported that the imidazole adduct with 1,1,1-trifluoroacetone resonates at 74 ppm (Liang & Abeles, 1987). If a sulfur nucleophile were considered, then measurements with $AACOCF_3$ and ethyl mercaptan indicate that resonances at or around 92.6 or 82.4 ppm might be anticipated.

DISCUSSION

The behavior of α-fluoroketones in solution is often complex (Figure 5). The ketone (**1**) can, in aqueous solution, add H_2O to form the hydrate (**2**); under basic conditions a proton can be abstracted to form the oxoanion (**4**). The hydrate can react with a nucleophile such as an alcohol to form the hemiketal derivative (**3**), and this species can be deprotonated similarly (**5**). It is this facile dynamic that makes this type of compound an excellent tetrahedral transition-state/reactive intermediate analog inhibitor of many different types of hydrolytic enzyme. Hydrated ketones are potent inhibitors of serine proteases (Imperiali & Abeles, 1986; Liang & Abeles 1987; Stein et al., 1987; Brady et al., 1990; Peet et al., 1990; Parisi & Abeles 1992). For this class of enzymes it has been demonstrated that a stable hemiketal is formed between the trifluoromethyl ketone inhibitor and the active site serine residue (Liang & Abeles 1987; Stein et al., 1987; Brady et

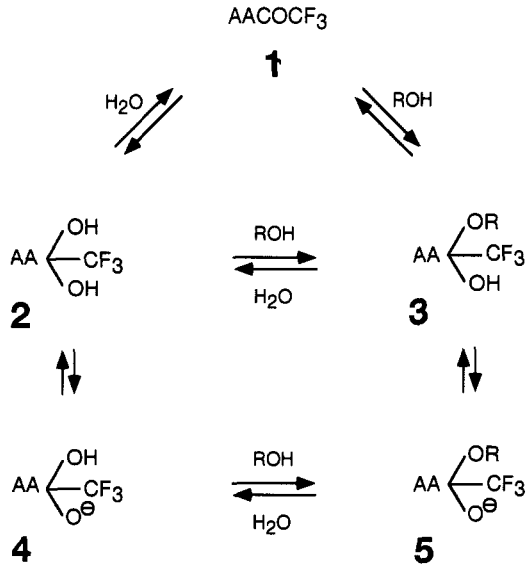


FIGURE 5: Equilibrium diagram showing the species of $AACOCF_3$ which interconvert in aqueous alcoholic solutions.

al., 1990; Parisi & Abeles, 1992). However, hydrated ketones are also potent inhibitors of zinc metalloproteases and aspartylproteases (Gelb et al., 1985). For both of these latter enzymes, it is probably the tetrahedral hydrate which is the inhibitory species since it is a structural mimic of the intermediate that is formed during the hydrolysis of substrates. The work described here was aimed at elucidating the type of complex that is formed between the $cPLA_2$ and $AACOCF_3$.

The results of the ^{19}F NMR experiments (Figures 1 and 2) establish that 1 mol of $AACOCF_3$ per mole of $cPLA_2$ can be bound tightly in the active site of the enzyme and that the tight complex is in slow exchange with free ligand. These results are in good agreement with previous kinetic experiments. In the absence of free calcium the $AACOCF_3$·$cPLA_2$ complex slowly dissociates to produce free and active enzyme (Street et al., 1993), and 1 mol of $AACOCF_3$ per mole of enzyme is required for complete inhibition of the enzyme activity (I. P. Street and M. H. Gelb, unpublished data).

More detailed information on the form of the $cPLA_2$·$AACOCF_3$ complex was obtained from ^{13}C NMR experiments with AA^*COCF_3. The 125.76-MHz ^{13}C NMR spectrum of $cPLA_2$ in aqueous buffer is shown in Figure 3A. The horizontal bars indicate the limits of where the inhibitor signal might appear as a ketone at low field or hydrate/hemiketal in the central region of the spectrum. Fortunately, there are no $cPLA_2$ signals in these regions that might obscure the inhibitor signal. Conformational changes induced by inhibitor binding could affect a number of signals from proximal residues, but these natural abundance signals would be of very low relative intensity and could not be mistaken as arising from AA^*COCF_3.

When a 1:1 complex was formed between AA^*COCF_3 and $cPLA_2$, the spectrum in Figure 3B was obtained (also see Figure 4A). The indicated peak at 101 ppm must be attributable to AA^*COCF_3 specifically bound to $cPLA_2$. When the labeled inhibitor was dissolved in CD_3OD/D_2O and the ^{13}C NMR spectra recorded over a range of pH values, the resonance from the ionized hemiketal was observed at 101 ppm. These data suggest that the signal from the $cPLA_2$·AA^*COCF_3 complex at 101 ppm arises from an enzyme-bound ionized hemiketal. Histidine or cysteine adducts can be excluded on the basis of chemical shift. After competition of the AA^*COCF_3 from the active site of the $cPLA_2$ with a large excess of $AACOCF_2Cl$, a new resonance at 94.5 ppm was observed. This corresponds to the nonspecifically bound AA^*COCF_3. On the basis of the titration data, this species can be assigned as the neutral hydrate of AA^*COCF_3.

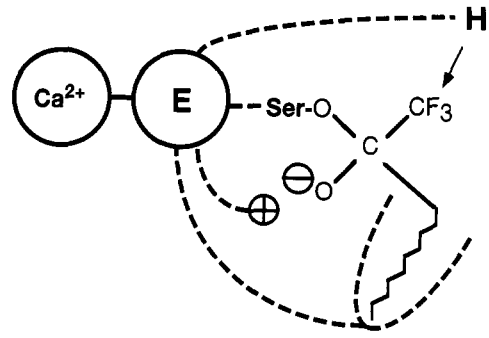


FIGURE 6: Model for $AACOCF_3$ bound tightly to $cPLA_2$.

However, because the environment of the active site as well as functionalization and ionization of the bound inhibitor can affect its resonance position, caution should be exercised in making structural assignments on the basis of chemical shift values alone. Spera and Bax (1991) have noted that the ^{13}C NMR chemical shifts of C_α and side-chain carbons of amino acid residues can be quite sensitive to protein secondary structure, and Torchia et al. (1988) have reported a chemical shift distribution of ca. 8 ppm for the alanyl methyl carbons in staphylococcal nuclease. However, it is unlikely that the bound AA^*COCF_3 exists substantially in the carbonyl form, since this species would be expected to resonate in the region of 190–200 ppm, and this is some 90–100 ppm downfield from the resonance observed from the specifically bound $AACOCF_3$ (101 ppm). Thus the tetrahedral structure of the labeled carbon in the enzyme–inhibitor complex can be assigned quite unambiguously since the chemical shift difference due to the functionalization of the labeled carbon is large when compared with any shift which might be expected from the protein environment. However, the monoanion of hydrated $AACOCF_3$ resonates at 97.2 ppm, and this value is reasonably close to the chemical shift of the specifically bound $AACOCF_3$. Thus the possibility that the enzyme-bound species might be the monoanion of the hydrate cannot be absolutely excluded, since, in this case, a further downfield shift of only 3.8 ppm must be attributed to the environment of the active site.

The results obtained here with $AACOCF_3$ and the $cPLA_2$ are very similar to those obtained previously with α-chymotrypsin and a trifluoromethyl ketone dipeptide analog (Liang & Abeles, 1987). In the study of α-chymotrypsin, the free inhibitor resonated at 96 ppm, and the signal due to the enzyme–inhibitor complex was observed at 104 ppm. On the basis of comparison with model compounds, the structure of the α-chymotrypsin–inhibitor complex was assigned as an ionized hemiketal formed between the bound inhibitor and the active site serine residue. The structural conclusions derived from the NMR study of α-chymotrypsin were later verified when an X-ray crystal structure of the enzyme–inhibitor complex was solved (Brady et al., 1990). In this study, the resonance position of the $cPLA_2$·$AACOCF_3$ complex is very similar to that of the α-chymotrypsin–trifluormethyl ketone complex, and thus the functionalization and the environment of the labeled carbon in the enzyme–inhibitor complexes of $cPLA_2$ and α-chymotrypsin are likely very similar. Therefore, by analogy with serine proteases we have postulated a model for the structure of the $AACOCF_3$·$cPLA_2$ complex (Figure 6). The enzyme is divided into Ca^{2+}-binding and catalytic domains. The carbon chain of the inhibitor is bound in a hydrophobic pocket, and the carbonyl of the

$AACOCF_3$ forms a covalent bond with a serine side chain, generating a hemiketal. The pK_a of a simple hemiketal of a trifluoromethyl ketone is approximately 9.1 (Liang & Abeles, 1987). We have concluded that the bound $AACOCF_3$ inhibitor exists as a charged hemiketal, and this suggests that its pK_a is lowered substantially in the active site. Salt bridge and/or hydrogen bond formation between the oxoanion of the hemiketal adduct and groups on the enzyme could account for this increase in acidity.

The results presented here are relevant only to the structure of an enzyme–inhibitor complex and do not provide direct evidence for the involvement of a serine group or an acyl–enzyme intermediate in the catalytic mechanism of the $cPLA_2$. However, the data are so strikingly similar to those obtained with α-chymotrypsin that it is tempting to speculate that there may also be similarities in the catalytic mechanisms of the two enzymes. The recent observation that the $cPLA_2$ is also able to catalyze transacylation reactions (Reynolds et al., 1993) led these authors to also tentatively propose the involvement of an acyl–enzyme intermediate in the catalytic pathway. Again the observation of transacylation is not a direct proof of the involvement of an acyl–enzyme intermediate; however, it is a characteristic reaction of many enzymes which do utilize this catalytic strategy. Further work is underway to obtain more definitive evidence for the involvement of an acyl–enzyme intermediate and to identify important amino acid residues.

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