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The binding of amide substrate analogues to phospholipase A_2

Studies by ^{13}C-nuclear-magnetic-resonance and infrared spectroscopy

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(*R*)-(2-dodecanamidoisohexyl)phosphocholine (DAHPC), labelled with ^{13}C at the amide carbonyl group, has been synthesized and its binding to bovine pancreatic phospholipase A_2 (PLA_2) studied by n.m.r. and i.r. spectroscopy. Two-dimensional ^1H-n.m.r. spectra show that, in the presence of Ca^{2+}, DAHPC binds to the active site of the enzyme in a similar manner to other phospholipid amide substrate analogues. The environment of the labelled carbonyl group has been investigated by a combination of ^{13}C n.m.r. and difference-Fourier-transform i.r. spectroscopy. The carbonyl resonance shifts 3 p.p.m. downfield on the binding of DAHPC to PLA_2. The carbonyl absorption frequency decreases by 14–18 cm^{-1}, accompanied by a marked sharpening of the absorption band. These results indicate that the carbonyl bond undergoes significant polarization in the enzyme–ligand complex, facilitated by the enzyme-bound Ca^{2+} ion. This suggests that ground-state strain is likely to promote catalysis in the case of substrate binding. Simple calculations, based on the i.r. data, indicate that the carbonyl bond is weakened by 5–9 $kJ \cdot mol^{-1}$. This is the first report of observation of the amide vibration of a bound ligand against the strong background of protein amide vibrations.

INTRODUCTION

The phospholipases A_2 (PLA_2s; EC 3.1.1.4) are a ubiquitous family of Ca^{2+}-dependent enzymes which catalyse the hydrolysis of fatty acids, particularly arachidonic acid, from the *sn*-2 position of aggregated glycerophospholipids (Bereziat *et al.*, 1990; Dennis *et al.*, 1991). Subsequent metabolism of the released arachidonic acid results in the formation of a number of eicosanoid products, including prostaglandins (Moncada & Higgs, 1988) and leukotrienes (Samuelsson *et al.*, 1987), which are believed to play a major role in the inflammatory response. The other product of the action of PLA_2 is a lysophospholipid, which can give rise to changes in the properties of biological membranes (Weltzien, 1979) and which can also be metabolized to platelet activating factor, another potent cellular mediator (Snyder, 1985).

Both membrane-associated and secreted forms of PLA_2 are present in, and produced by, cells participating in the inflammatory reaction. To date, the most widely studied PLA_2s are the low-molecular-mass ($\sim$ 14 kDa), Ca^{2+}-dependent extracellular enzymes (Dennis *et al.*, 1991). Members of this family have now been purified from a wide range of sources, including bee and snake venom, mammalian pancreas, rheumatoid arthritic synovial fluid (Loeser *et al.*, 1990) and are also secreted by other mammalian cell types such as platelets (Kramer *et al.*, 1989).

High-resolution structures determined by X-ray crystallography have been available for several years for PLA_2 from bovine (Dijkstra *et al.*, 1981) and porcine (Dijkstra *et al.*, 1983) pancreas, and for the dimeric enzyme from rattlesnake venom (Brunie *et al.*, 1985). More recently, crystal structures of the enzyme and its inhibitor complexes from human rheumatoid arthritic synovial fluid (Scott *et al.*, 1991; Wery *et al.*, 1991), from bee and snake venom (Scott *et al.*, 1990*b*; White *et al.*, 1990) and of a mutant porcine pancreatic enzyme (Thunnissen *et al.*, 1990) have been reported. We and others have been using two-dimensional n.m.r. to probe the structure of, and molecular recognition by, the mammalian pancreatic PLA_2s. Substantial numbers of resonances have been assigned in the spectra of the bovine (Fisher *et al.*, 1989; W. U. Primrose, H. Kogelberg & G. C. K. Roberts, unpublished work) and porcine (Dekker *et al.*, 1991) pancreatic enzymes; the assigned resonances include virtually all of the hydrophobic residues which form the substrate-binding pocket. Recently we have used n.m.r. data, principally inter- and intra-molecular nuclear Overhauser effects (NOEs) to define the location and orientation of some amide substrate analogues in the active site of PLA_2 in solution (Bennion *et al.*, 1992).

The mechanism of the enzyme has long been assumed to be analogous to that of the serine proteinases on the basis of similarities between their active sites (Verheij *et al.*, 1980; Kamer & Argos, 1981). There is a histidine–aspartate couple of the correct geometry to facilitate nucleophilic attack of a water molecule on the scissile ester bond of the substrate [although it should be noted that the geometry of this couple differs significantly from that in the serine proteinases (Kamer & Argos, 1981)], and the anionic transition state could be stabilized by the close proximity of the essential Ca^{2+} ion and the backbone amide of Gly-30. This mechanism has received substantial support from recent crystallographic studies of the binding of a phosphonate 'transition-state analogue' inhibitor to the bee- and snake-venom enzymes, and a detailed proposal for the mechanism has been advanced (Scott *et al.*, 1990*a*). An important feature of this mechanism is the postulated role of the essential

Abbreviations used: PLA_2, phospholipase A_2; F.t.i.r., Fourier-transform i.r.; DAHPC, (*R*)-(2-dodecanamidoisohexyl)phosphocholine; NOE(SY), nuclear-Overhauser-effect (spectroscopy); TOCSY, total correlation spectroscopy.

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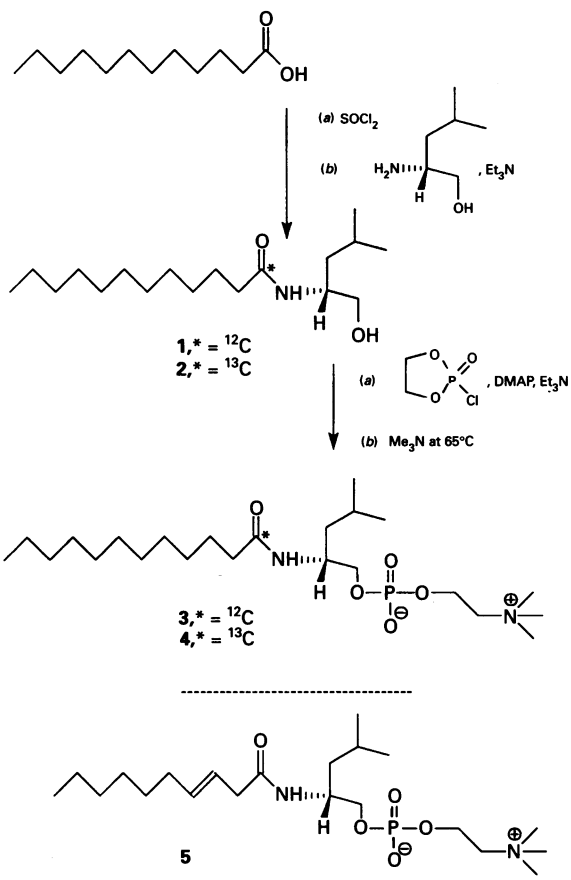
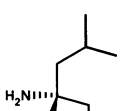


Scheme 1. Synthesis of DAHPC

Formula	Definition	Abbreviation or formula	Definition
		$SOCl_2$	Thionyl chloride
	*R*-Leucinol	Et_3N	Triethylamine
		DMAP	4-Dimethyl-aminopyridine
	2-Chloro-2-oxo-1,3,2-dioxaphospholane	Me_3N	Trimethylamine

Ca^{2+} ion both in substrate binding, through interactions with the phosphate and with the carbonyl group of the scissile ester bond, and in catalysis, through stabilization of the oxyanion in the transition state. Here we present the results of studies of the environment of the carbonyl group of an amide substrate analogue, (*R*)-(2-dodecanamidoisohexyl)phosphocholine (DAHPC; **3** and **4**; see Scheme 1), in the active site of the enzyme, by ^{13}C-n.m.r. and Fourier-transform i.r. (F.t.i.r.) spectroscopy.

EXPERIMENTAL

Synthesis of inhibitor

Acetonitrile, benzene and triethylamine were dried by distillation from calcium hydride. Routine ^1H-n.m.r. spectra were recorded at 360 MHz on a Bruker AM360 spectrometer. Mass spectra were recorded on a VG 70-250 SEQ instrument, and elemental analyses were performed by using a Perkin–Elmer 240B instrument. Optical rotations were measured on an AA-1000 polarimeter, and melting points (uncorrected) were determined with a Buchi melting-point apparatus.

The synthetic route is outlined in Scheme 1. A solution of 1.3 mmol of dodecanoic acid or [*carboxyl*-^{13}C]dodecanoic acid (obtained from Aldrich) in thionyl chloride (5 ml) was refluxed for 1 h, and residual thionyl chloride was removed *in vacuo*. A solution of the acid chloride in dichloromethane (5 ml) was added to a stirred solution of *R*-leucinol (0.15 g, 1.3 mmol) and triethylamine (0.13 g, 1.3 mmol) in dichloromethane (5 ml). The solution was stirred for 1 h at room temperature, and was then treated with 2 M-HCl (5 ml). The organic phase was removed, washed with 10% $NaHCO_3$ (2 × 5 ml), dried over anhydrous $MgSO_4$ and evaporated *in vacuo* to give the amido alcohol.

(*R*)-(*N*)-[(1-Hydroxymethyl)isopentyl]dodecanamide (compound **1**, Scheme 1): yield 90%; m.p. 70–73 °C; *m/z* 300 [$(M+H)^+$, 100%]; δ_H [(^2H)chloroform]: 0.88 (3H, t, *J* 5.7 Hz, $CH_3[CH_2]_{10}$), 0.94 [6H, dd, *J* 5.7 Hz, $CH(CH_3)_2$], 1.2–1.4 (19H, m, $[CH_2]_9CH_3$, $CH(CH_3)_2$), 1.6 [2H, m, $CH_2CH(CH_3)_2$], 2.20 (2H, t, *J* 5.7 Hz, CH_2CONH), 2.98 (1H, bs, O*H*), 3.54 [1H, dd, *J* 11, 5.7 Hz, C*H*H(OH)], 3.68 [1H, dd, *J* 11, 2.9 Hz, CH*H*(OH)], 4.05 (1H, m, NHC*H*) and 5.58 (1H, d, *J* 6.9 Hz, N*H*) p.p.m.

(*R*)-(*N*)-[1-Hydroxymethyl)isopentyl]-[1-^{13}C]dodecanamide (**2**):yield 75%; *m/z* 301 [$(M+H)^+$, 100%]; ^1H-n.m.r. spectrum as for **1**.

A solution of 2-chloro-2-oxo-1,3,2-dioxaphospholane (90 mg, 0.63 mmol) in benzene (0.5 ml) was added to a stirred suspension of the amido alcohol (0.5 mmol) and 4-dimethylaminopyridine (20 mg, 0.15 mmol) in benzene (10 ml) under N_2 at room temperature. The suspension was stirred for 5 h, filtered, and the filtrate evaporated *in vacuo*. The residue was dissolved in acetonitrile (2 ml) and treated with 3 M-trimethylamine in acetonitrile (10 mmol) at 65 °C for 15 h. The solvent was removed by evaporation and the residue was purified by silica column chormatography (eluent chloroform/methanol/water, 65:25:4, by vol.) to give DAHPC.

DAHPC (**3**): yield 52%; $[\alpha]_D^{25}$ +20±1° (*c* 0.6, methanol); elemental analysis: found: C, 54.65; H, 10.31; N, 5.75; $C_{23}H_{49}N_2O_5P,2.4H_2O$ requires: C, 54.40; H, 10.68; N, 5.52%; *m/z* 465 [$(M+H)^+$, 100%]; δ_H [(^2H)chloroform] 0.89 {9H, m, $[CH_2]_9CH_3$ and $CH(CH_3)_2$}, 1.2–1.3 (18H, m, $[CH_2]_9CH_3$), 1.42 [1H, m, $CH(CH_3)_2$], 1.60 [2H, m, $CH_2CH(CH_3)_2$], 2.18 (2H, m, CH_2CONH), 3.33 [9H, s, $N(CH_3)_3$], 3.79 (4H, m, CH_2N, OCH_2), 4.1 (1H, m, NHC*H*), 4.28 (2H, m, CH_2OP) and 7.25 (1H, d, *J* 5.8 Hz, N*H*) p.p.m.

[1-^{13}C]DAHPC (**4**); yield 42%; *m/z* 466 [$(M+H)^+$, 100%]; ^1H-n.m.r. spectrum as for **3**.

Sample preparation

Bovine pancreatic phospholipase A_2 was prepared from pancreas as described by (Dutilh *et al.*, 1975). Samples for spectroscopy contained 1–2 mM-enzyme and 1–2 mM-inhibitor as appropriate. Samples were made up in either (i) Ca^{2+}-free buffer, which was 300 mM-NaCl/250 μM-EDTA/10 mM-[$^2H_{11}$]Tris (Aldrich), pH* 7.6 in ^2H_2O (99.96%; Goss Scientific Instruments, Ingatestone, Essex, U.K.) or (ii) Ca^{2+}-containing buffer, which was 50 mM-$CaCl_2$/200 mM-NaCl/10 mM-[$^2H_{11}$]Tris, pH* 7.6 in ^2H_2O. Solution conditions for each experiment are given in the Figure legends. The notation pH* denotes a pH-meter reading uncorrected for the deuterium-isotope effect on the glass electrode.

F.t.i.r. spectroscopy

F.t.i.r. spectra were recorded using a Nicolet 60SX spectrometer equipped with a Nicolet 1280 computer and with a mercury–cadmium telluride type A detector. The instrument was purged continuously with dry air to maintain a very low water-

vapour pressure (−70 °C dew point); the sample cell (CaF_2, 50 μm pathlength) was filled and emptied from outside the instrument in order to maintain the purge. For each spectrum 1000 scans were averaged (500 ms/scan) at a resolution of 2 cm^{-1}.

N.m.r. spectroscopy

^1H N.m.r. spectra were recorded at 500 MHz on a Bruker AM500 spectrometer. Two-dimensional nuclear-Overhauser-effect spectroscopy (NOESY) spectra were recorded with the time-proportional-phase-increment method (Wider *et al.*, 1984). Total correlation spectroscopy (TOCSY) spectra were recorded using an MLEV-17 spin-lock field (Bax & Davis, 1985). All two-dimensional data were recorded with 512 data points in f_1 and 2048 data points in f_2. Before Fourier transformation, the data were zero-filled in f_1 and multiplied by a Gaussian window function. ^{13}C n.m.r. spectra were recorded at 150 MHz on a Bruker AMX600 spectrometer. All spectra were recorded at 314K.

RESULTS AND DISCUSSION

^1H-n.m.r. studies of the mode of inhibitor binding

Before focusing on the environment of the amide carbonyl group, it is important to establish the way in which DAHPC binds to the enzyme. We have recently studied the complex of PLA_2 with a closely related amide inhibitor, (*E*)-(*R*)-(2-dec-3-eneamidoisohexyl)phosphocholine (**5**), having a $C_{10}(\Delta^{3,4})$ hydrocarbon chain in place of the saturated C_{12} chain of DAHPC, and have used intermolecular NOEs involving the olefinic protons of the inhibitor, in combination with molecular modelling, to derive a structure for the complex (Bennion *et al.*, 1992). In the case of DAHPC itself, we have not been able to assign individual methylene proton resonances of the bound inhibitor, and hence we cannot use intermolecular NOEs in the same way. However, inhibitor binding is accompanied by characteristic changes in chemical shifts and NOEs for a number of resonances assigned to residues in the binding site, and a comparison of the shift changes produced by DAHPC with those observed previously for **5** provides a sensitive means of establishing whether the two compounds bind to the enzyme in the same way.

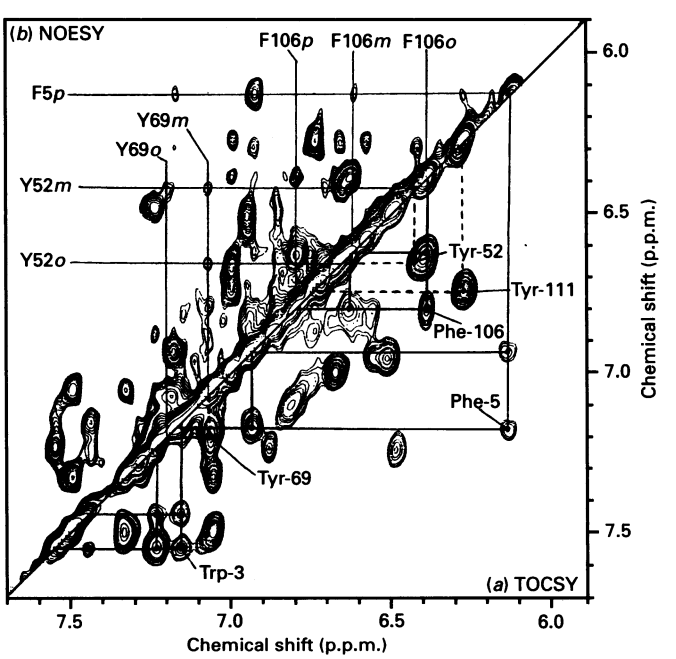


Fig. 1. Two-dimensional ^1H-n.m.r. spectra of the complex of DAHPC (3) and PLA_2 in the presence of calcium

The aromatic region of the 500 MHz homonuclear ^1H two-dimensional TOCSY (*a*, bottom right) and NOESY (*b*, top left) spectra of 2 mM bovine pancreatic PLA_2 in Ca^{2+}-containing buffer (see the Experimental section) with 2 mM-DAHPC. The spin systems for Phe-5, Phe-106, Tyr-52, Tyr-69, Tyr-111 and Trp-3 are shown on the TOCSY spectrum. The expected positions of cross-peaks corresponding to inter-residue contacts between Tyr-52 and Tyr-69, and between Phe-5 and Phe-106, are shown on the NOESY spectrum. The suffices *o*, *m* and *p* refer to the C^δ, C^ε and C^ζ protons respectively.

Fig. 1 shows the aromatic resonance correlations of the 500 MHz ^1H–^1H TOCSY and NOESY spectra of the DAHPC–Ca^{2+}–PLA_2 complex, and the chemical shifts of some relevant resonances are summarized in Table 1, where they are compared with the shifts in the spectra of the enzyme alone and of the complex with compound **5** (all recorded in the presence of saturating Ca^{2+} concentrations). Extensive resonance assignments have been made in the spectra of the enzyme alone and of its complexes with other inhibitors (Fisher *et al.*, 1989; Bennion *et al.*, 1992; W. U. Primrose, H. Kogelberg & G. C. K. Roberts, unpublished work). The resonances of the enzyme–DAHPC complex were assigned by detailed comparison of the TOCSY and NOESY patterns seen for this complex with those seen in the previously assigned spectra.

It is clear that the effects of DAHPC and **5** on the ^1H-n.m.r. spectrum of the protein are very similar. For the 18 resonances listed in Table 1, the average absolute difference in chemical shift between the two complexes is 0.03 p.p.m. compared with an average change from the native enzyme of 0.12 p.p.m. For seven of these resonances, from Trp-3, Phe-5, Tyr-52, Tyr-69, Phe-106 and Tyr-111, the change in shift on the binding of DAHPC is more than 0.10 p.p.m. Differences of greater than 0.05 p.p.m. between the two complexes are seen for only three resonances in Table 1, from Phe-5, Phe-106 and Tyr-111. The two phenylalanine residues are close to the $C^3{=}C^4$ double bond in the complex of compound **5** with the enzyme, as shown by the observation of NOEs between the olefinic protons of the inhibitor and the aromatic protons of these two residues (Bennion *et al.*, 1992); the absence of this double bond in DAHPC will affect the chemical shifts of the nearby residues, owing to the absence of its magnetic anisotropy effects, and there may also be small local differences in geometry. Tyr-111 lies close to, but not in, the binding site, and the behaviour of its resonance reflects a local conformational equilibrium in the enzyme which is influenced by pH, temperature, Ca^{2+} binding (Fisher *et al.*, 1989) and inhibitor binding (W. U. Primrose, unpublished work). There may thus be a slight difference in this equilibrium between the two complexes, although it should be noted that the C^δ methyl resonances of Leu-41, which is close to Tyr-111 in the structure and which is affected by the same equilibrium, behave very similarly in the two cases.

A further point of similarity between the spectra of the two complexes is the observation of three strong NOEs between the aromatic protons of Tyr-52 and Tyr-69 (Fig. 1). These NOEs reflect a movement of Tyr-69 on inhibitor binding, across the mouth of the binding pocket, its phenolic hydroxy group becoming hydrogen-bonded to the inhibitor phosphate. In addition, both enzyme–inhibitor complexes show a similar reduction in the number and intensity of NOEs between Phe-5 and Phe-106 compared with those that can be seen in the native enzyme (Fisher *et al.*, 1989). These two phenylalanine residues comprise part of the wall of the hydrophobic binding pocket and could be expected to move apart on the binding of the alkyl chain of the inhibitors into this pocket.

We can thus conclude that DAHPC binds to the enzyme in a

Table 1. ^{1}H-n.m.r. chemical shifts of selected residues of bovine PLA_2 alone and in its complex with DAHPC (3) and compared with its complex with compound 5

Buffer conditions: 2 mM-PLA_2, with or without 1.2 mol-equiv. of DAHPC (3), in Ca^{2+}-containing buffer.

	Chemical shift† (p.p.m.)		Chemical-shift difference‡ (p.p.m.)	
Proton*	δ_E (Enzyme alone)	$\delta_{E\cdot DAHPC}$ (enzyme·DAHPC complex)	Δ_E (from enzyme alone)	Δ_5 (from complex with 5)
Trp-3 $C^{\epsilon 3}H$	7.60	7.56	−0.04	−0.01
$C^{\zeta 3}H$	7.27	7.16	−0.11	−0.03
$C^{\eta}H$	7.32	7.25	−0.07	−0.01
$C^{\zeta 2}H$	7.51	7.45	−0.06	−0.03
Phe-5 $C^{\delta}H$	7.10	7.19	+0.09	+0.03
$C^{\epsilon}H$	6.96	6.93	−0.03	−0.03
$C^{\zeta}H$	6.34	6.14	−0.20	−0.06§
Ile-9 $C^{\delta}H_3$	0.02	−0.06	−0.08	0.00
Leu-41 $C^{\delta}H_3$	0.06	0.13	+0.07	+0.03
	0.45	0.52	+0.07	+0.02
Tyr-52 $C^{\delta}H$	6.71	6.67	−0.04	+0.01
$C^{\epsilon}H$	6.30	6.43	+0.13	+0.03
Tyr-69 $C^{\delta}H$	7.16	7.21	+0.05	−0.02
$C^{\epsilon}H$	6.81	7.08	+0.27	−0.01
Phe-106 $C^{\delta}H$	6.41	6.39	−0.02	+0.09§
$C^{\epsilon}H$	6.87	6.64	−0.23	+0.02
$C^{\zeta}H$	7.20	6.82	−0.38	−0.04
Tyr-111 $C^{\delta}H$	6.70	6.76	+0.06	+0.05§
$C^{\epsilon}H$	6.13	6.28	+0.15	+0.07§

* Protons whose chemical shifts are included are those with assigned resonances which shift appreciably on binding of DAHPC.
† Chemical shifts are expressed in p.p.m. downfield from 5,5-dimethylsilapentanesulphonate and were measured relative to the $C^{\alpha}H$ resonance of Cys-77 at 5.56 p.p.m.
‡ $\Delta_E = \delta_{E\cdot DAHPC} - \delta_E$; $\Delta_5 = \delta_{E\cdot DAHPC}$ − (chemical shift in complex with compound 5).
§ Significantly different between the complexes of 3 and 5 (see the text).

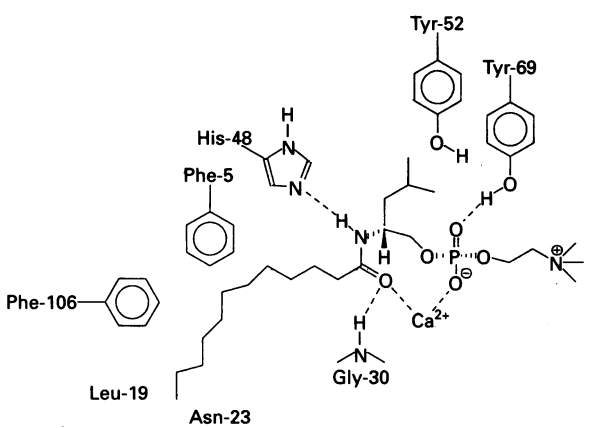


Fig. 2. Binding of DAHPC (3) to PLA_2

The expected binding of DAHPC within the active site of bovine pancreatic PLA_2 is based on the derived structure of the complex of compound 5 with the enzyme (Bennion *et al.*, 1992). The hydrogen-bonding pattern around the amide of the ligand is shown, as well as the positions of four aromatic residues whose n.m.r. signals are perturbed by the binding.

very similar fashion to compound 5, for whose complex we have earlier proposed a model (Bennion *et al.*, 1992). This is in turn very similar to the structure of the complex between a mutant porcine pancreatic PLA_2 and the amide inhibitor (*R*)-(2-dodecanamidohexyl)phosphoglycol which has been determined by X-ray crystallography (Thunnissen *et al.*, 1990). The proposed mode of binding of DAHPC to the bovine enzyme is shown in Fig. 2, where it can be seen that the carbonyl group of the amide interacts with the essential Ca^{2+} and with the backbone NH of Gly-30. In addition, it is proposed that the amide nitrogen donates a hydrogen bond to the neutral imidazole of His-48. This additional interaction appears to be of particular importance to the formation of the enzyme–inhibitor complex, since protonation of the histidine considerably reduces affinity (Yu & Dennis, 1991).

In seeking to use spectroscopic methods in solution to study the effects of binding on the amide carbonyl group of the inhibitor, which might throw light on the environment of the scissile bond of the substrate, it is necessary to be able to identify the spectroscopic signals from this specific carbonyl separately from those of the more than 100 amide carbonyl groups in the protein. For both n.m.r. and i.r. spectroscopy, this can be done most readily by isotopic substitution with ^{13}C; DAHPC enriched to 90% in ^{13}C at the amide carbonyl group has therefore been synthesized as described in the Experimental section.

^{13}C-n.m.r. studies of the carbonyl environment

Fig. 3 shows the 150 MHz ^{13}C n.m.r. spectra of [^{13}C]DAHPC (4) in buffer, and in the presence of PLA_2, with and without Ca^{2+}. the carbonyl ^{13}C resonance of the free inhibitor appears at 174.0 p.p.m., and on addition of the enzyme with no Ca^{2+} it is hardly changed at 174.4 p.p.m. The inhibitor binds only weakly and non-specifically to the enzyme in the absence of Ca^{2+} (as indicated by the ^{1}H-n.m.r. spectrum under these conditions; results not shown). In the presence of Ca^{2+}, however, there is a substantial downfield shift of the carbonyl resonance to 177.1 p.p.m. The relative linewidths of the resonance (Fig. 3) show that only in the presence of Ca^{2+} is the inhibitor tightly bound to the active site of the enzyme; the slight increase in linewidth in the presence of enzyme, but without Ca^{2+}, can be attributed to the

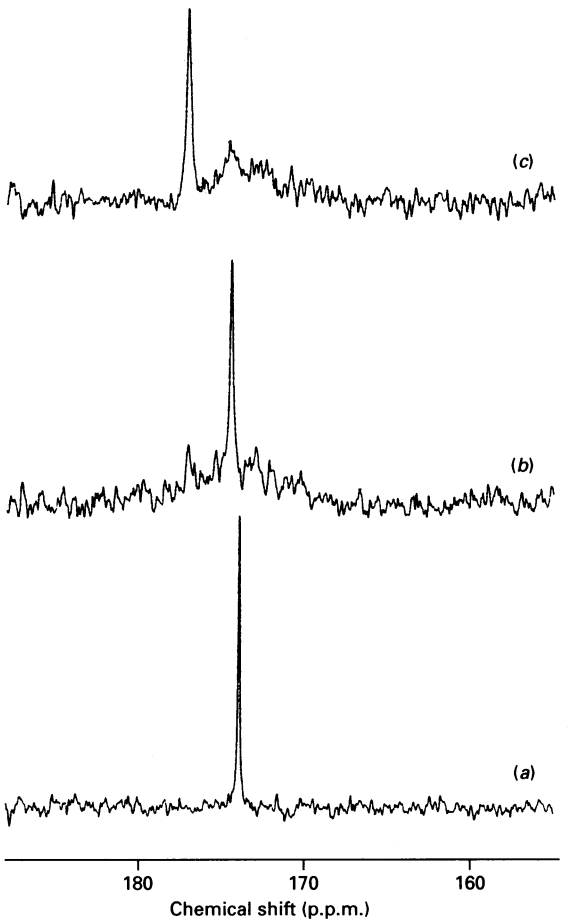


Fig. 3. ^{13}C-n.m.r. spectra of the binding of DAHPC (3) to PLA_2

150 MHz ^{13}C-n.m.r. spectra of compound **4**, with and without bovine pancreatic PLA_2 are shown. All spectra were obtained with 2 mM-compound **4**. In (*a*) and (*b*) Ca^{2+}-free buffer was used; in (*c*) Ca^{2+}-containing buffer was used. In (*b*) and (*c*) 2 mM-enzyme was present. Chemical shift (p.p.m.) and linewidth (Hz) for the enriched resonance in each case are: (*a*) 174.0, 3.4; (*b*) 174.4, 17.2; (*c*) 177.1, 35.7. Spectra were transformed after 5 Hz line-broadening and are referenced to internal dioxane at 66.2 p.p.m.

increased viscosity of the solution due to the high concentration of protein and to weak non-specific binding. The 3 p.p.m. downfield shift on binding in the presence of Ca^{2+} is, first of all, not consistent with any significant degree of tetrahedral character at the amide carbonyl group of the bound ligand; this would lead to a large shift in the opposite direction. The downfield shift (deshielding) of the carbonyl resonance is in the direction expected for a polarization of the C=O bond such that the electron density on the oxygen atom is increased and that on the carbon atom decreased. When the inhibitor is examined in aqueous solution, the carbonyl group is presumably involved in hydrogen-bonding interactions with water molecules (as indicated by F.t.i.r.; see below), and the downfield shift on binding to the enzyme therefore reflects an increase in the polarization of the C=O bond over that already produced by these interactions with water molecules. Given the proposed structure of the complex, this additional polarization can be largely attributed to an interaction with the essential Ca^{2+} ion. Previous workers have shown that small downfield shifts in carbonyl resonances can be observed when appropriately labelled small protein inhibitors are complexed with serine proteinases (Baillargeon *et al.*, 1980; Richarz *et al.*, 1980). In those cases, the shifts are of the order of 0.7–0.9 p.p.m. These reflect the difference between the H-bonding to the carbonyl group from two water molecules in the free ligand, and the H-bonding from two backbone amides in the bound species. The difference between free and bound carbonyl resonances is much larger for DAHPC and PLA_2, and reflects the much greater degree of polarization of the amide carbonyl that is possible in this situation as opposed to that in the serine proteinases. Since ester hydrolysis by PLA_2 cannot proceed through an acyl-enzyme intermediate, as is the case with the serine proteinases, the increased polarization observed here may be required in order to drive the reaction forward, since water is not covalently attached to the enzyme (unlike serine in the serine proteinases) and the entropic requirements will thus be more severe. Presumably this is also reflected in an enhanced stabilization of the tetrahedral oxyanion intermediate.

Previous workers have tried to correlate the downfield shift in a carbonyl ^{13}C n.m.r. signal with a change in bond order. The binding of oxaloacetate to citrate synthase produces a downfield shift of between 4.3 and 6.8 p.p.m. in the carbonyl resonance, depending on the chemical nature of the third component of the ternary complex (Kurz *et al.*, 1985). Those authors propose that such a change corresponds to a decrease in carbonyl bond order from 2.0 to 1.7 (Tiffon & Dubois, 1978) or to 20–30% protonation of the carbonyl oxygen atom. They concede that such quantification based solely on observed changes in chemical shift is open to question, but further evidence, based on F.t.i.r. measurements, appears to support their initial conclusions (Kurz & Drysdale, 1987). In the present work, similar calculations are complicated by two other possible contributions to the observed change in chemical shift: from the magnetic anisotropy of nearby aromatic rings and from electron redistribution resulting from hydrogen-bonding to the amide nitrogen. Calculations based on published crystal structures, and on the model shown in Fig. 2 for DAHPC binding, indicate that the shift contributions from nearby aromatic rings will be small. Studies on small molecules indicate that the effects on the carbonyl resonance position of amide nitrogen hydrogen-bonding by water are small (Eaton & Symonds, 1988; Eaton *et al.*, 1989). The effect of the hydrogen bond to an imidazole group (as shown in Fig. 2) cannot be estimated with any precision, but its contribution to the total shift is likely to be upfield. The observed 3 p.p.m. downfield shift is therefore likely to be an underestimate of the carbonyl polarization effect. If we neglect the other small contributions, the observed shift corresponds to a reduction in the carbonyl bond order from 2.0 to > 1.8. This calculation is clearly subject to some error; nonetheless, it shows that there is clearly substantial ground-state substrate-analogue destabilization by carbonyl polarization (see also the F.t.i.r. results presented below). Such a change in bond order could contribute greatly in energetic terms to the binding, but the increased carbon–oxygen bond length [< 0.005 nm (< 0.05 Å)] would be unlikely to be visible in a crystallographic structure.

Infrared studies of the carbonyl environment

In using i.r. spectroscopy to study changes in the environment of carbonyl groups in ligands on binding to enzymes, difference spectra in which free enzyme spectra are subtracted from spectra of the complex are complicated by the presence of features which arise from perturbations of groups in the protein on ligand binding (White & Wharton, 1990). Features which arise from changes in both carboxy group (~ 1700 cm^{-1}) and backbone amide (1620–1680 cm^{-1}) absorptions have been observed previously for phospholipase A_2 in complexes with substrate analogues (Kennedy *et al.*, 1990). Difference spectra in which the spectrum of a complex with a [^{13}C]carbonyl-labelled ligand is subtracted from that of the complex with the same ligand with an isotopically normal [^{12}C]carbonyl group are unaffected by such

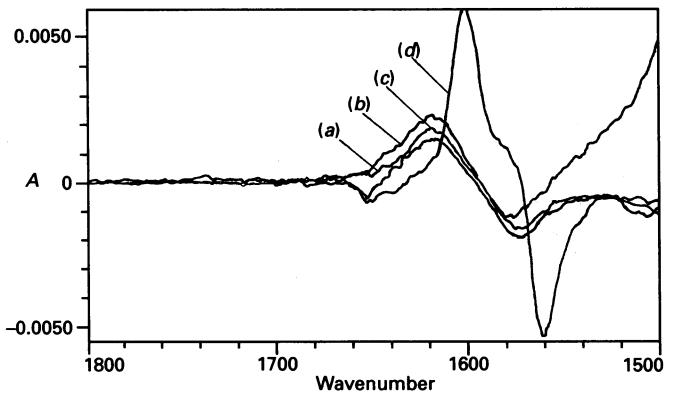


Fig. 4. F.t.i.r. difference spectra of DAHPC in the presence or absence of both Ca^{2+} and PLA_2

Spectra shown are subtractions of the spectrum for **4** ($^{13}C{=}O$) from that for **3** ($^{12}C{=}O$). All spectra were obtained with 1 mM-**3** (-**4**). In (*a*) and (*c*) Ca^{2+}-free buffer was used; in (*b*) and (*d*) the buffer contained Ca^{2+}. In (*a*) and (*b*) no enzyme was present; in (*c*) and (*d*) 1 mM bovine pancreatic PLA_2 was present.

Table 2. Characteristics of the i.r. absorptions of the carbonyl group of DAHPC (3) and [^{13}C]DAHPC (4) free and bound to bovine PLA_2.

Data are from spectra in Fig. 4, except that measured in dichloromethane (CH_2Cl_2) (results not shown); bandwidths were measured at half-maximum absorbance; n.m., not measured. DAHPC and (when present) PLA_2 concentrations were 1 mM.

Inhibitor	PLA_2	Ca^{2+}	Frequency (cm^{-1})	Peak absorbance (m*A*)	Bandwidth (cm^{-1})
[^{12}C]DAHPC in CH_2Cl_2			1658	n.m.	n.m.
(3)	−	−	1619	1.54	33.2
	−	+	1620	2.37	34.7
	+	−	1620	1.90	29.1
	+	+	1602	5.96	17.2
[^{13}C]DAHPC in CH_2Cl_2			1617	n.m.	n.m.
(4)	−	−	1573	1.88	32.1
	−	+	1575	1.63	28.9
	+	−	1579	1.22	20.1
	+	+	1561	5.26	16.3

perturbations of the protein spectrum (Tonge *et al.*, 1991) and have been used here for this reason.

Difference F.t.i.r. spectra of this kind are shown in Fig. 4, for DAHPC in buffer and when bound to PLA_2, in the presence and absence of Ca^{2+}; the band frequencies, intensities and linewidths are summarized in Table 2. In these difference spectra, the overlap of the absorption bands of the ^{13}C and ^{12}C species can lead to a larger frequency difference between the two maxima than the 38 cm^{-1} calculated theoretically from the reduced masses. In the present case, the observed frequency difference is 41 cm^{-1}, indicating that this distortion is small in these experiments.

Addition of equimolar PLA_2 to the solution of the inhibitor in the absence of calcium led to only small changes (1–6 cm^{-1}) in frequency of the i.r. absorption of the carbonyl group of the inhibitor. Addition of Ca^{2+} to the aqueous solution of the free inhibitor had no significant effect on the i.r. absorption, but when Ca^{2+} was added in the presence of PLA_2, there was a decrease in absorption frequency of 14–18 cm^{-1}, together with a marked sharpening of the absorption band. This decrease in frequency, like the change in ^{13}C resonance frequency discussed above, clearly indicates a polarization of the carbonyl bond on binding to the enzyme in the presence, but not in the absence, of Ca^{2+}. The decrease in bandwidth, for which there is ample precedent in other systems (Belasco & Knowles, 1980, 1983; White & Wharton, 1990), can be attributed to a decrease in conformational dispersity or mobility of the ligand in the complex. Examination of the Fourier-deconvoluted and second-derivative spectra (not shown) of the bound ligands reveals that the main bands represent a single species in each case. Each ligand is thus bound in a single conformation.

Comparison of the frequencies of the carbonyl absorption of the free ligand in buffer and dichloromethane (Table 2) shows that hydrogen-bonding from water contributes a frequency shift of 38–44 cm^{-1}. We can assume that the free ligand has two water molecules hydrogen-bonded to the carbonyl oxygen atom. Very approximately, then, the frequency shift on complexation with the enzyme is equivalent to a further hydrogen bond from water. The 14–18 cm^{-1} frequency shift may be related to a change in the carbonyl (double) bond energy by simple, though approximate, calculations. The frequency in wavenumbers is proportional to the vibrational photon energy, and one may assume that, for small changes in frequency, the corresponding change in bond energy is proportional to the change in vibrational energy. On this assumption, the change in carbonyl bond energy (for $^{12}C{=}O$) on binding to the enzyme in the presence of Ca^{2+} can be estimated as approx. $[(14\text{–}18\ cm^{-1})/1620\ cm^{-01}] \times 800\ kJ \cdot mol^{-1} = 7\text{–}9\ kJ\ mol^{-1}$, where the energy of the carbonyl bond is taken as 800 $kJ \cdot mol^{-1}$. A somewhat more complex calculation, based on an analysis that assumes simple harmonic motion for the C=O vibration (White & Wharton, 1990), yields a value of 5 $kJ \cdot mol^{-1}$. Given the approximate nature of the calculations, the agreement between these estimates is reasonable. They indicate that the polarization of the carbonyl bond of the inhibitor on binding represents a significant weakening of the bond.

It is notable that the integrated intensity (area) of the carbonyl absorption is increased by a factor of approx. 1.5 when the inhibitor is bound to the enzyme in the presence of Ca^{2+}. A similar effect was seen when oxaloacetate was bound to citrate synthase, with a further enhancement of intensity on formation of a ternary complex (Kurz & Drysdale, 1987). Although a frequency shift of 21 cm^{-1} occurred on oxaloacetate binding, there was no further frequency shift on ternary-complex formation. Thus it has been proposed that intensity enhancement, as seen here, is an additional indicator of carbonyl hydrogen bonding and the associated strain.

The magnitude of the frequency shift on binding seen in the present work compares with a carbonyl shift of 19 cm^{-1} seen when dihydroxyacetone binds to triosephosphate isomerase (Belasco & Knowles, 1980) [not seen with a mutated non-functional enzyme (Komives *et al.*, 1991)], a 24 cm^{-1} shift when glyceraldehyde phosphate binds to aldolase (Belasco & Knowles, 1983) and a 29 cm^{-1} shift between acyl carbonyl groups in productively and non-productively bound dihydrocinnamate in the form of an acyl-enzyme with chymotrypsin (White & Wharton, 1990). The binding of the inhibitor in the active site of PLA_2 in this case produces a shift of 14–18 cm^{-1}. In comparison, the change in the frequency on changing the solvent from non-hydrogen-bonding (dichloromethane) to hydrogen-bonding (water) for the inhibitor alone is 38–42 cm^{-1} (Table 2). If we assume that the carbonyl oxygen atom is hydrogen-bonded to two water molecules in free solution, when it interacts with the Ca^{2+} ion and with the backbone NH of Gly-30, then there is further polarization of the carbonyl similar to that produced by

one additional good hydrogen bond. In the case of substrate binding, polarization may be predicted to promote catalysis by ground-state strain (cf. White & Wharton, 1990). However, it is clear from recent crystallographic work (Scott *et al.*, 1990*b*, 1991; Thunnissen *et al.*, 1990; White *et al.*, 1990) that, although the carbonyl group of the inhibitor makes the same contacts with the enzyme as the P=O group of a phosphonate transition-state analogue, the precise mode of binding of the amide bond of the inhibitor must differ appreciably from that of the ester bond of a substrate. The complex of the particular inhibitor used here, and those of amide substrate analogues in general, appear to have some resemblance to a product complex. The hydrogen bond from the active-site histidine residue corresponds to the situation during hydrolysis, where a proton is about to be transferred to the (alcohol) leaving group. This contributes an additional amount of stabilization to the enzyme–inhibitor complex, which would have not been present if only the oxyanion hole had been filled. Thus although we can conclude from the experiments reported here that ground-state strain is likely to contribute to catalysis by PLA_2, we cannot give a precise estimate of the magnitude of this contribution.

The F.t.i.r. study reported here shows, for the first time, that it is possible to observe the amide vibrations of bound ligands against the strong background of the enzyme amide vibrations. Previous studies have been restricted to a region of the spectrum (> 1690–$1750\ cm^{-1}$) where protein amide absorption is weak or absent. By use of the isotope-difference method, clear-cut interpretation of the spectral features that arise from the bound ligand has been possible. This form of experiment may be of general utility, especially for small proteins, where the background absorption will be of manageable proportions.

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