# Changes in the structure of bovine phospholipase $A_2$ upon micelle binding

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Phospholipase $A_2$ ($PLA_2$) is a calcium-dependent enzyme which hydrolyses the 2-acyl ester bond of phospholipids. The extracellular $PLA_2$s are activated by as much as 10000-fold on binding to micelles or vesicles of substrate, possibly due to a conformational change induced in the enzyme. We have studied the complex of bovine pancreatic $PLA_2$ with micelles of SDS by ultracentrifugation, equilibrium dialysis, microcalorimetry, fluorescence and n.m.r. spectroscopy. Ultracentrifugation and equilibrium dialysis measurements showed that on average 1.28 ($\pm$0.17) $PLA_2$ molecules and 26.4 ($\pm$3.1) SDS molecules are involved in the complex and that there is a rapid equilibrium between micellar species containing one or more enzyme monomers. The estimated heat of formation of the complex, measured calorimetrically as the heat released when $PLA_2$ was injected into excess 10 mM SDS, was 162.3 ($\pm$1.5) kJ/mol [38.8 ($\pm$0.35) kcal/mol] of $PLA_2$ added. The fluorescence of the single tryptophan at position 3 in the N-terminal helix of the protein increases when $PLA_2$ binds to SDS micelles, indicating that this part of the protein is in a more hydrophobic environment in the complex. The structural changes in $PLA_2$ on addition of [$^2H_{25}$]SDS were monitored using n.m.r. spectroscopy. The overall structure of the protein is unchanged, but changes in nuclear Overhauser effects (NOEs) were observed for residues in the N-terminal helix, at the active site region and in a lysine-rich region near the C-terminus. The NOE changes at the N-terminus indicate that this portion of the protein molecule adopts a more ordered, helical conformation when bound to a micelle. We suggest that these conformational changes could be the mechanism by which the enzyme becomes activated in the presence of aggregated substrate.

## INTRODUCTION

Phospholipase $A_2$ ($PLA_2$) is a calcium-dependent enzyme which hydrolyses the 2-acyl ester bond of phospholipids to release fatty acids and lysophospholipids [1–4]. The fatty acids released, most notably arachidonic acid, have a role in inflammation as the precursors of prostaglandins and leukotrienes [5], while the other products, lysophospholipids, are the precursors of platelet aggregating factor and other physiologically important compounds. $PLA_2$ is therefore a potential target for anti-inflammatory drugs.

The extracellular $PLA_2$s, such as the pancreatic and platelet enzymes, hydrolyse aggregated substrates up to 10000-fold faster than monomeric substrates. Several theories for the mechanism of this dramatic activation have been proposed [1,6]. (a) The interfacial recognition site model. This model suggests that the enzyme interacts with the substrate micelle through a specific interface recognition site distinct from the active site. This induces a conformation change in the enzyme which includes a reorientation of the active-site residues and thereby increased catalytic activity. (b) The dual phospholipid model. This model, originally proposed for snake venom phospholipases, suggests that a single activating phospholipid molecule interacts with a site on the enzyme which is distinct from the active site. This affects the structure of the active site of another enzyme molecule which is the catalytic partner in an enzyme dimer. Since two phospholipid molecules with different roles are involved this model is called the 'dual phospholipid' model. (c) The substrate model. This model proposes that the enzyme remains constant in structure and that the enhancement of catalytic activity arises from the fact that phospholipids in micelles are constrained to conformations which favour binding and hydrolysis. (d) Product-release mechanism. In this model it is proposed that the hydrophobic product diffuses out of the active site more easily into a micelle than into aqueous solution. If product-release is the rate-determining step (which is not known) then micelle binding will enhance the rate of phospholipid hydrolysis.

A good deal of structural information on $PLA_2$ and its complexes with monomeric substrate analogues is available from X-ray crystallography [6–8], but there is much less information on the interaction of the enzyme with aggregated substrate or substrate analogues. Two recent studies have provided evidence for conformational changes in $PLA_2$ on micelle binding. A study of $PLA_2$ fluorescence concluded that the internal motion of Trp-3 was considerably reduced and that the flexibility of the calcium-binding loop was increased upon binding of the enzyme to micelles [9]. Studies of porcine pancreatic phospholipase $A_2$ by n.m.r. showed changes in the conformation of the N-terminus and the active site upon formation of a ternary complex of enzyme, substrate analogue and micelle [10]. In the work reported here, we show that bovine pancreatic $PLA_2$ binds to SDS to form protein–SDS aggregates. SDS can be considered a simple, albeit imperfect, analogue of the anionic phospholipids which are the preferred substrates for the enzyme [11]. N.m.r. studies of these complexes show changes in the enzyme structure around the N-terminal region, the active site and a region close to the C-terminus. This conformational change may cause the enzyme to be activated upon binding to lipid micelles.

## MATERIALS AND METHODS

### Protein preparation

Bovine $PLA_2$ was prepared from pancreas by a modification of the literature procedures [12]; the final cation-exchange chromatography was replaced by chromatography on a Pharmacia

Abbreviations used: $PLA_2$, phospholipase $A_2$; $C_{19}$-ABK-$PLA_2$, phospholipase $A_2$ modified with 1-bromononadecanone; $C_{17}$-ABK-$PLA_2$, phospholipase $A_2$ modified with 1-bromoheptadecanone; RLM-1, 3-thiooctadecanyl-2-(R)-acetylaminophosphatidylcholine; NOE, nuclear Overhauser effect; p.p.m., parts per million.

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Hi-Load S column with 10 mM acetate buffer, pH 5.0, and a gradient of 0–250 mM sodium chloride to elute the protein. The protein was eluted at about 30 mM sodium chloride.

### Ultracentrifugation

Ultracentrifugation was carried out on a Beckman Optima XLA ultracentrifuge using scanning u.v. optics. The protein concentrations were measured by absorbance at 280 nm [$A_{280}$ (1 %) = 13.0]. All the runs were carried out at 20 °C at either 18000 or 40000 rev. min$^{-1}$. Samples of $PLA_2$ (0.75 mM, 10 mg/ml) were dialysed against 50 mM SDS/20 mM succinate/1 mM EDTA, pH 6.5, and then diluted to 72.5 $\mu$M either with the dialysis buffer or with the dialysis buffer lyophilized and reconstituted in $^2H_2O$, to generate a set of samples with $^2H_2O$ proportions of 5, 25, 50, 60, 80 and 95 % $^2H_2O$. The densities of these buffers were determined at 20 °C. The partial molar specific-volume of $PLA_2$ was calculated from the amino acid composition to be 0.730. The ultracentrifuge data were analysed to determine the apparent molecular mass of the complex. Data were fitted to the equation

$$c(r) = c_a \exp[(r^2 - r_a^2)\omega^2 M_p(1-\phi'\rho)/2RT]$$

where $r$ is the distance from the centre of the rotor, $c$ the protein concentration, $\omega$ the angular velocity, $M_p$ the mass of the protein in the complex, $\phi'$ the partial specific volume of the complex and $\rho$ the solvent density; $c_a$ and $r_a$ are the concentration at and the position of the first data-point used in the fit. The values of $M_p(1-\phi'\rho)$ and $c_a$ were treated as variables in non-linear regression using PROC NLIN in the SAS [13] statistical package. The derived values of $M_p(1-\phi'\rho)$ were plotted against $\rho$ and the data fitted by linear regression, weighted by the reciprocals of the variances of the $M_p(1-\phi'\rho)$ values. $M_p$ was determined from the value of $M_p(1-\phi'\rho)$ at the solvent density corresponding to the reciprocal of the partial specific volume of SDS. The $\phi'$ value of the $PLA_2$–SDS complex was obtained from the intercept on the $x$-axis, where $\phi' = 1/\rho$.

### Equilibrium dialysis

Equilibrium dialysis was carried out in cells of 100 $\mu$l volume on each side of the dialysis membrane. The membrane was 10000 Da cut-off dialysis tubing from Spectra/Por (Pierce and Warriner, Chester, U.K.). One side of the cell contained 75 $\mu$l of $PLA_2$ solution (0.2 mM enzyme in 20 mM succinate/1 mM EDTA, pH 6.5), and the other the same volume of 50 mM SDS in the same buffer. [$^{35}$S]SDS (Amersham) was added to the 50 mM SDS so that the solution gave about 6000 c.p.m./$\mu$l (200 Bq, $6 \times 10^{-7}$ Ci) upon scintillation counting. The equilibrium dialysis cells were left at 37 °C for 1 week in a shaking incubator for equilibrium to be reached. Control cells containing buffer alone in one compartment were dialysed against SDS to measure the attainment of equilibrium. Samples of 10 $\mu$l of liquid from each side of the dialysis cell were removed and diluted to 5 ml with scintillation fluid (Optiphase 'Safe' from LKB) and counted for 1 min to determine the difference in SDS concentrations on each side of the membrane. This difference was the amount of SDS bound to the $PLA_2$. Leakage of protein through the membrane was checked for by measurement of the $A_{280}$ of solutions on each side of the membrane.

### Microcalorimetry

Microcalorimetry experiments were carried out on a Microcal titration calorimeter at 25 °C with a cell volume of 1.4115 ml. $PLA_2$ was in 20 mM Mes buffer/1 mM EGTA, pH 6.5. To determine the heat of complexation of $PLA_2$ to an excess of SDS, five injections of 50 $\mu$l of 0.398 mM $PLA_2$ were made into a solution of 10 mM SDS in the same buffer as the protein. A control addition of buffer into SDS was also performed. To determine the heat change when SDS was added to $PLA_2$, 25 injections of 10 $\mu$l of 100 mM SDS were made into 0.391 mM $PLA_2$. A control addition of SDS into buffer was also carried out and the heat changes subtracted from the first titration. A similar pair of titrations was conducted with 25 mM SDS and 0.356 mM $PLA_2$.

### Determination of the effect of SDS on the activity of $PLA_2$

A solution of $PLA_2$ (0.182 mM) and SDS (50 mM) in 20 ml total volume was left at room temperature for 1 h. The specific activity of this solution was measured by the pH stat assay using egg-yolk phospholipids [14] and compared with the specific activity of the original $PLA_2$ solution.

### Fluorescence experiments

Fluorescence spectra were obtained on a Perkin-Elmer LS-5B luminescence spectrometer. The fluorescence cell was either a $4 \times 4 \times 25$ mm, 250 $\mu$l volume quartz cell or a $3 \times 3 \times 5.4$ mm (49 $\mu$l) cell in which 60 $\mu$l of solution was used to avoid bubbles in the cell windows. All buffer solutions were filtered through 0.2 $\mu$m Acrodiscs (Gelman Sciences) before use. All experiments were carried out at 25 °C. Fluorescence excitation was at 295 nm with a 2.5 nm excitation slit-width. The fluorescence emission was measured over the range 305–450 nm with a 2.5 nm emission slit-width; for titrations, emission was measured at 340 nm with a 5 nm slit-width. Titrations were performed with solutions of 1–20 mM lipid. Hexadecylphosphocholine (20 mM) was added in aliquots to solutions of bovine or porcine $PLA_2$ (5 $\mu$M) in Tris buffer at pH 8.0 (50 mM Tris/0.1 M KCl/25 mM $CaCl_2$) and the fluorescence measured after each addition. A control titration was carried out without the $PLA_2$ and these data were subtracted from the fluorescence intensities in the first titration. Solutions of SDS (1–100 mM) were added to $PLA_2$ (5 $\mu$M) in various buffers, all at 20 mM concentration; Tris was used at pH 8.0, Hepes at pH 7.4 and Mes at pH 6.0.

### N.m.r. spectroscopy

Samples of $PLA_2$ for n.m.r. were prepared at 0.5–4 mM concentrations. For experiments in $^2H_2O$ the pH of the samples was adjusted to pH 7.4 or 6.1 (uncorrected pH meter readings) and lyophilized from 99.9 % $^2H_2O$ at least twice. These samples were then redissolved in 99.99 % $^2H_2O$ (400 $\mu$l, from Goss Scientific) and transferred to 5 mm n.m.r. tubes. Calcium-containing samples were made up with 50 mM $CaCl_2$/0.2 M NaCl and either 10 mM Tris, pH 7.4, or 20 mM $^2H_4$-succinate, pH 6.1. Samples for experiments with SDS were made up with the same buffers without $CaCl_2$ but with 0.25 mM EDTA. For experiments in $H_2O$, 10 % $^2H_2O$ was added to act as a field-frequency lock signal and the buffer was 20 mM [$^2H_4$]succinate, pH 6.5. Titration with [$^2H_{25}$]SDS was carried out by addition of microlitre quantities of 0.7 M or 0.1 M solutions of SDS dissolved in $^2H_2O$.

N.m.r. spectra were obtained by using either a Bruker AM500 spectrometer with an Oxford Instruments 11.7 T magnet or a Bruker AMX600 spectrometer with an Oxford Instruments 14 T magnet. One-dimensional spectra in $^2H_2O$ were acquired with presaturation of the residual water resonance for 1 s with about 2 W of radiofrequency power. For experiments in $H_2O$, weaker presaturation was combined with a 'jump-return' acquisition

pulse [15]. A 6 kHz spectral width was used for spectra recorded in $^2H_2O$, and 10 kHz for spectra recorded in water; a pulse-width of about 8 $\mu$s (70°) was used and 8192 real data-points were collected.

Two-dimensional spectra were acquired with 2048 real data-points in $\omega_2$ and 512 increments in $\omega_1$; 64–128 scans were acquired per increment so that each spectrum took 12–24 h to acquire. Sine modulation was used in the acquisition of phase-sensitive data using the time proportional phase incrementation (TPPI) method [16]. NOE spectroscopy (NOESY) and double-quantum-filtered correlation spectroscopy experiments were performed using standard methods [16–18]. Total correlation spectroscopy spectra were acquired using the MLEV-17 spin-lock sequence [19] and spin-lock times of 30–80 ms. Rotating frame Overhauser enhancement spectroscopy experiments were carried out using the main transmitter for spin-locking pulse and spin-lock times of 30–70 ms [20]. The spin-locking field was 7.7 kHz. Data were zero-filled in the $\omega_1$ dimension to 1024 data-points and multiplied by a Gaussian window-function before two-dimensional Fourier transformation. Spectra in $H_2O$ were baseline-corrected using Bruker software. Chemical shifts are reported relative to 2,2-dimethyl-2-silapentane-5-sulphonate.

### Preparation of α-bromoketone-modified $PLA_2$s

#### Synthesis of 1-bromononadecan-2-one

Diazomethane was prepared from Diazald (27 mmol, 5.8 g, from Aldrich) using the standard procedure. Half of the diazomethane solution (about 10 mmol, in 40 ml ether) was cooled on ice and stirred. Stearoyl chloride (2 mmol, 0.606 g, from Aldrich) was dissolved in anhydrous ether (20 ml) and added dropwise to the diazomethane. The reaction mixture was stirred overnight at room temperature. The product absorbed u.v. light, stained with phosphomolybdic acid spray and had an $R_F$ of 0.7 in ether on t.l.c. on silica gel. No starting material was observed on t.l.c. The ether and unreacted diazomethane were removed by a stream of dry nitrogen, the solid was redissolved in dry ether (100 ml) and hydrogen bromide gas was bubbled through for several minutes. The ether was removed under vacuum and the solid dissolved in ethyl acetate (100 ml). This was extracted with water (5 × 200 ml) until the washings were no longer acid. The organic layer was dried with sodium sulphate and reduced under vacuum to give a crystalline solid. T.l.c. of this solid in ether showed a spot of $R_F$ 0.8 which was not u.v. absorbing and stained with phosphomolybdic acid spray. This solid was 1-bromononadecan-2-one and was used without purification in the subsequent reactions. $\delta$ (p.p.m.) [($^2$H)chloroform] 0.89 (3H, t, $J$ 9 Hz, $CH_3$), 1.32–1.62 ( ~ 27H, m, $(CH_2)_{15}$), 2.63 (2H, $J$ 12 Hz, $CH_2CO$), 3.87 (2H, s, $CH_2Br$). Mass spectrum $m/z$ [electron impact (EI)]): 362/360, 1 % $M^+$ ($^{79}$Br and $^{81}$Br); 361/359, 2 % $(M-H)^+$, ($^{79}$Br and $^{81}$Br); 281, 10 %, $(M-Br)^+$; 267, 100 %, $C_{17}H_{35}CO^+$.

#### Synthesis of 1-bromoheptadecan-2-one

In the same way, palmitoyl chloride (2 mmol, 0.55 g, Aldrich) was reacted with diazomethane and subsequently with hydrogen bromide gas to produce 1-bromoheptadecan-2-one. This and the diazo intermediate had the same properties on t.l.c. as the products derived from stearoyl chloride. $\delta$ (p.p.m.) ([$^2$H]chloroform) 0.85 (3H, t, $J$ 9 Hz, $CH_3$), 1.30–1.60 [ ~ 25H, m $(CH_2)_{13}$], 2.64 (2H, t, $J$ 11 Hz, $CH_2CO$), 3.86 (2H, s, $CH_2Br$). Mass spectrum (E.I.) $m/z$ 334/332, < 1 % $M^+$ ($^{79}$Br and $^{81}$Br); 333/331, < 1 % $(M-H)^+$ ($^{79}$Br and $^{81}$Br); 253, 4.3 % $(M-Br)^+$; 239, 100 %, $C_{15}H_{31}CO^+$.

#### Inactivation of $PLA_2$ with α-bromoketones

1-Bromoheptadecan-2-one (50 $\mu$l, 0.1 M in acetone) was added to $PLA_2$ (18 mg) in 4 ml of 60 mM phosphate buffer/2 mM EGTA/15 mM SDS, pH 7.0, and stirred at 37 °C. (SDS was found to be necessary for the reaction to proceed; presumably it solubilizes the long-chain α-bromoketone.) After 12 h a further 50 $\mu$l of 1-bromoheptadecan-2-one solution was added and the reaction left for a further 24 h. The mixture was then dialysed against two changes of phosphate buffer (2 litres, 10 mM, pH 7.0) for 2 days. The pH of this material was lowered to 4.5 and it was loaded onto a Dynachrom CS[4] cation-exchange column equilibrated with acetate buffer (10 mM, pH 5.0). A salt gradient (0–200 mM NaCl) was run in this buffer and the modified protein was eluted at about 50 mM salt concentration. The modified protein was collected, concentrated in an Amicon concentrator and 5.37 mg of protein obtained for n.m.r. $PLA_2$ (15.4 mg) was inactivated in the same way with 1-bromononadecan-2-one to yield 4.05 mg of modified protein after chromatography.

## RESULTS

Bovine pancreatic $PLA_2$ was determined to have a specific activity of 96 $\mu$mol·min$^{-1}$·mg$^{-1}$ in the standard assay [14] using egg-yolk phospholipids. After incubation for 1 h at room temperature in 50 mM SDS, the specific activity was measured as 117 $\mu$mol·min$^{-1}$·mg$^{-1}$; within experimental error, these values are the same. This clearly indicates that the protein retains an active conformation in the presence of SDS micelles. Preliminary n.m.r. experiments confirmed that $PLA_2$ retains a folded conformation under these conditions, and also showed changes in the spectrum indicative of the formation of a complex between the enzyme and SDS. We therefore set out to characterize this complex in detail.

### Composition of the complex

The ultracentrifugation method of Reynolds and Tanford [21] was used to determine the molecular mass of the protein component of the SDS complex. A plot of $M_p(1-\phi'\rho)$ against the density of the solvent, $\rho$, is shown in Figure 1. This extrapolates

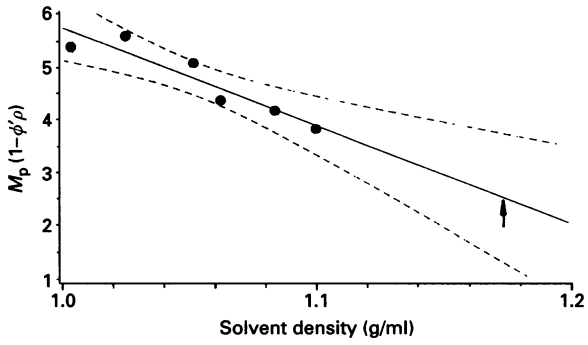


**Figure 1 Determination of the molecular mass of the protein component of the $PLA_2$–SDS complexes using ultracentrifugation**

$PLA_2$ (72.5 $\mu$M) in 5–95% $^2H_2O$ solutions was centrifuged in the presence of SDS. The parameter $M_p(1-\phi'\rho)$, determined from a curve fitted to the change in protein concentration along the centrifuge tube, is shown as a function of solvent density. The arrow indicates the position where the effect of the SDS is balanced by the solvent and $M_p$ can be determined, since $\rho$ is known and the partial specific volume of the protein alone can be used for $\phi'$. The broken lines indicate the 95% confidence limits for the linear regression.

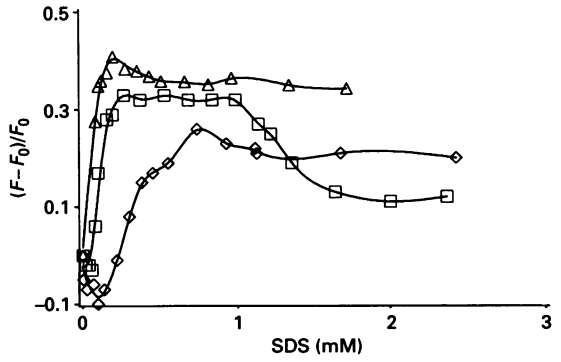


**Figure 2 Fluorescence change upon addition of SDS to $PLA_2$**

Aliquots of SDS were added to 5 $\mu$M $PLA_2$ and the fluorescence monitored at 340 nm with excitation at 295 nm. The data are shown as fractional fluorescence change, $\Delta F/F_0$. □, pH 6.0; △, pH 7.4 and ◇, pH 8.0.

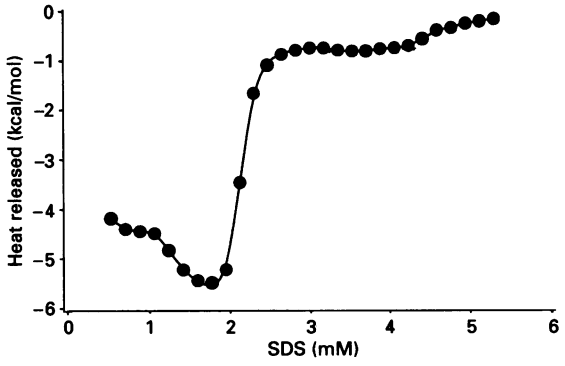


**Figure 3 Calorimetric study of the interaction of SDS with $PLA_2$**

Aliquots of 25 mM SDS solution (10 $\mu$l) were injected into 1.4115 ml of a 0.356 mM solution of $PLA_2$ in the sample cell of a microcalorimeter. The data show the heat released as a function of SDS concentration, after correction for the heat of dilution of SDS into buffer.

to a molecular mass of 17.6 ± 2.4 kDa for the protein component of the SDS–$PLA_2$ complex. A second set of data on a different set of $PLA_2$ samples gave 17.3 ± 0.4 kDa as the molecular mass of the protein component in the complex.

This ultracentrifugation method can in principle also be used to determine the partial molar specific-volume, $\phi'$, of the complex and hence its overall composition. However, the combination of experimental error with the long extrapolation to the $x$-axis used to determine $\phi'$ for the complex led to large uncertainties in the estimate of the number of SDS bound per $PLA_2$ molecule. At a concentration of 50 mM SDS the number of SDS molecules bound per $PLA_2$ was calculated by this method to be 27 (± 14).

A more precise estimate of the amount of SDS in the complex was obtained by equilibrium dialysis of $PLA_2$ in the presence of 50 mM SDS (using tracer [$^{35}$S]SDS). This gave a value of 20.6 (± 2.0) molecules of SDS bound per $PLA_2$ molecule. Using the ultracentrifugation estimate of protein mass per complex, the protein–SDS complex therefore contains on average 26.4 (± 3.1) molecules of SDS and 1.28 (± 0.17) protein molecules.

### Binding curves: fluorescence and calorimetry

Fluorescence has been used to study the binding of $PLA_2$ to micelles of several lipid analogues [22]. In agreement with earlier observations [23], we have confirmed that addition of hexadecylphosphocholine to $PLA_2$ (5 $\mu$M) at pH 8.0 led to an increase in tryptophan fluorescence intensity measured at 340 nm, together with a blue shift of the emission maximum from 350 nm to less than 340 nm. These titrations showed a smooth and continual increase of fluorescence as the lipid was added.

Addition of increasing concentrations of SDS to a solution of $PLA_2$ at pH 6–8 produced complex changes in the tryptophan fluorescence of the protein, as shown in Figure 2. (The experiment was repeated in the presence of 2 mM EDTA to eliminate the effects of any residual calcium, with essentially identical results.) At low SDS concentrations ( < 0.2 mM) there is a small decrease in fluorescence emission, clearly visible at pH 8 and just discernible at pH 7.4 but not apparent at pH 6. This may correspond to the binding of an SDS molecule to the active site of the enzyme (see below). The initial decrease is followed at higher SDS concentrations by an increase in fluorescence of about 30–40%, which we ascribe to the formation of SDS–protein micellar complexes. This increase occurs at lower SDS concentrations at pH 6 and 7.4 than at pH 8, which may account for the differences in apparent amplitude of the initial decrease at the different pH values. Finally, the fluorescence decreases again to a variable extent at around 1 mM (the critical micellar concentration of SDS in these buffers in the absence of enzyme), indicating some change in the structure of the complex.

The binding-curve obtained on adding SDS to a solution of $PLA_2$ and measuring the heat released using a microcalorimeter is shown in Figure 3. The substantial heat change shows clearly that there is an interaction between $PLA_2$ and SDS, but again the binding-curve is complex. In particular, the finding that the heat released increases with increasing SDS concentration up to about 1.8 mM SDS indicates cooperativity of the kind to be expected for micelle formation. The heat released when $PLA_2$ was injected into excess 10 mM SDS was 162.3 (± 1.5) kJ/mol [38.8 (± 0.35) kcal/mol] of $PLA_2$ added. The heat changes observed in these experiments take place over a different range of SDS concentrations to the fluorescence changes shown above. This is due to the much higher concentrations of $PLA_2$ used in the calorimetry experiments. It is the ratio of SDS to $PLA_2$ which is important. If calorimetry were possible with the same concentrations of $PLA_2$ used in the fluorescence studies we would expect to see heat changes in the same concentration range.

### Structural changes: n.m.r. spectroscopy

The effects of addition of increasing concentrations of [$^2H_{25}$]SDS on the $^1$H n.m.r. spectra of $PLA_2$ are shown in Figures 4(a) and (b). Figure 4(a) shows that at concentrations of up to 2 mM SDS the linewidths of the resonances increased with only slight changes in chemical shifts. By 10 mM SDS the sample appeared slightly cloudy. On further increasing the SDS concentration (Figure 4b), the sample became clear and there was a transition to a different spectrum with narrower lines (narrower than in the absence of SDS) and a number of differences in chemical shift. Over the concentration range 10–35 mM SDS, the changes in the spectrum, for example in the high-field methyl region, showed behaviour characteristic of a slow exchange process. The differences in chemical shift observed (for resonances resolved in one- or two-dimensional spectra) between zero and 50 mM SDS are listed in Table 1. The largest chemical shift changes, > 0.5 p.p.m., are in resonances of Leu-2 and Trp-3. In addition, the methyl resonance of Ala-1, which is not observable in the spectrum of the enzyme alone, appears at −0.07 p.p.m. in the presence of SDS.

There are also clear changes in the NOESY spectrum on

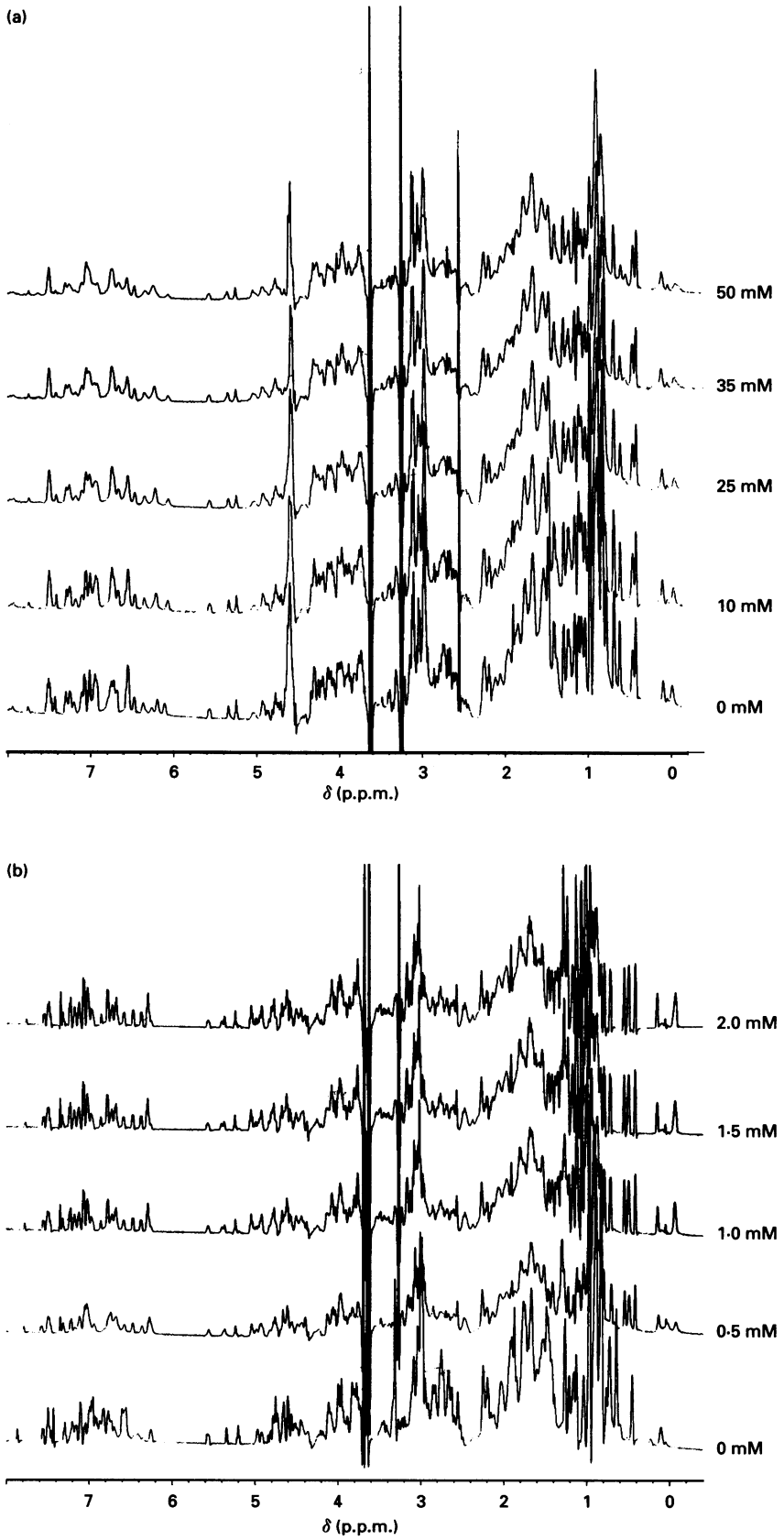


**Figure 4 Changes in the 500 MHz $^1$H-n.m.r. spectra of $PLA_2$ upon addition of [$^2H_{25}$]SDS: (a) 0–2 mM SDS at 314 K; and (b) 10–50 mM SDS at 330 K**

Samples contained 2 mM $PLA_2$ in 0.2 M NaCl, 20 mM [$^2H_4$]succinate, pH 6.1. Microlitre volumes of 0.1 M or 0.7 M [$^2H_{25}$]SDS in $^2H_2O$ were added.

**Table 1 Changes in $^1H$ chemical shift of residues of $PLA_2$ on addition of 50 mM SDS**

Chemical shift differences between $PLA_2$ in 0.2 M NaCl/50 mM $CaCl_2$/20 mM $^2H_4$-succinate, pH 6.1, and in 0.2 M NaCl/50 mM SDS/0.25 mM EDTA/20 mM $^2H_4$-succinate, pH 6.1. Positive values indicate that the resonance is further downfield in the presence of SDS than in its absence. Only those residues whose chemical shift changes by more than $\pm 0.02$ p.p.m. on addition of SDS are included in the Table. Assigned resonances which showed no chemical shift changes of more than $\pm 0.02$ p.p.m. were from Cys-27, Asp-39, Cys-84, Ala-93, Phe-94, Asn-97 and His-115. $C_xH$ indicates an aromatic proton from Tyr-28.

| Residue | Proton, $\delta$ (p.p.m.) | | | | |
|---|---|---|---|---|---|
| Leu-2 | $C_\gamma H$, 0.54 | $C_\delta H_3$, 0.20 | | | |
| Trp-3 | $C_\alpha H$, 0.08 | $C_\beta H_2$, 0.04 | $C_{\delta 1}H$, −0.15 | $C_{\zeta 3}H$, −0.15 | $C_{\zeta 2}H$, 0.52 |
| | | | $C_{\epsilon 3}H$, −0.12 | $C_{\eta 2}H$, −0.13 | $N_{\epsilon 1}H$, 0.06 |
| Gln-4 | $C_\alpha H$ −0.06 | | | | |
| Phe-5 | $C_\zeta H$, −0.08 | | | | |
| Met-8 | $C_\epsilon H$, 0.05 | | | | |
| Ile-9 | NH, 0.03 | $C_{\gamma 1}H$, 0.06 | $C_\gamma H_3$, 0.14 | $C_\delta H$, −0.09 | |
| Lys-12 | NH, −0.05 | $C_\alpha H$, 0.03 | | | |
| Ile-13 | $C_\beta H$, 0.06 | | | | |
| Phe-22 | $C_\delta H$, 0.07 | | | | |
| Tyr-25 | $C_xH$, 0.03 | | | | |
| Gly-26 | NH, 0.17 | $C_\alpha H$, 0.03 | | | |
| Tyr-28 | NH, −0.15 | $C_xH$, −0.20 | | | |
| Cys-29 | NH, −0.37 | | | | |
| Ser-34 | NH, −0.04 | $C_\alpha H$, 0.07 | | | |
| Thr-36 | NH, −0.07 | $C_\alpha H$, 0.04 | | | |
| Pro-37 | $C_\alpha H$, 0.05 | | | | |
| Val-38 | NH, 0.17 | $C_\beta H$, 0.04 | $C_{\gamma 1}H$, 0.03 | | |
| Leu-41 | NH, −0.11 | $C_\gamma H$, 0.08 | $C_{\delta 1}H_3$, 0.09 | $C_{\delta 2}H_3$, 0.04 | |
| Asp-42 | NH, −0.12 | $C_\alpha H$, 0.15 | | | |
| Arg-43 | NH, −0.10 | | | | |
| Cys-44 | NH, 0.04 | | | | |
| Cys-45 | NH, −0.06 | | | | |
| Gln-46 | NH, −0.13 | $C_\alpha H$, −0.03 | | | |
| Thr-47 | CH, −0.03 | $C_\beta H$, −0.07 | $C_\gamma H$, −0.03 | | |
| His-48 | NH, 0.04 | $C_{\epsilon 1}H$, −0.09 | $C_{\delta 2}H$, 0.23 | | |
| Asp-49 | NH, 0.10 | | | | |
| Asn-50 | NH, −0.10 | | | | |
| Tyr-52 | $C_\delta H$, −0.05 | | | | |
| Ala-55 | NH, −0.04 | $C_\alpha H$, 0.16 | $C_\beta H_3$, 0.04 | | |
| Leu-58 | $C_\alpha H$, −0.11 | $C_{\delta 2}H_3$, −0.08 | | | |
| Tyr-69 | $C_\delta H$, 0.06 | $C_\epsilon H$, 0.26 | | | |
| Tyr-73 | NH, 0.09 | $C_\alpha H$, 0.09 | | | |
| Ser-74 | NH, −0.08 | | | | |
| Tyr-75 | NH, −0.03 | $C_\alpha H$, 0.04 | $C_\delta H$, 0.07 | $C_\epsilon H$, 0.14 | |
| Ser-76 | NH, −0.06 | | | | |
| Cys-77 | $C_\beta H_2$, 0.03, 0.04 | | | | |
| Glu-81 | NH, 0.06 | | | | |
| Ile-82 | NH, −0.16 | $C_\alpha H$, 0.08 | $C_\beta H$, 0.04 | $C_\delta H$, −0.09 | $C_\gamma H$, 0.04 |
| Thr-83 | NH, −0.04 | | | | |
| Ser-85 | NH, 0.03 | | | | |
| Asn-89 | NH, 0.06 | $C_\alpha H$, −0.03 | | | |
| Ala-90 | $C_\alpha H$, −0.05 | $C_\beta H_3$, −0.03 | | | |
| Glu-92 | NH, 0.09 | | | | |
| Ile-95 | NH, −0.12 | $C_\alpha$, −0.03 | | | |
| Cys-96 | NH, 0.05 | | | | |
| Cys-98 | NH, −0.27 | | | | |
| Asp-99 | NH, 0.23 | | | | |
| Arg-100 | NH, 0.05 | | | | |
| Asn-101 | NH, −0.06 | | | | |
| Ala-102 | NH, −0.22 | $C_\alpha H$, −0.03 | | | |
| Ala-103 | $C_\alpha H$, 0.05 | $C_\beta H_3$, 0.04 | | | |
| Ile-104 | NH, −0.06 | | | | |
| Cys-105 | NH, −0.08 | | | | |
| Phe-106 | NH, −0.08 | $C_\delta H$, −0.03 | $C_\epsilon H$, −0.07 | | |
| Ser-107 | NH, −0.08 | | | | |
| Tyr-111 | $C_\delta H$, 0.08 | $C_\epsilon H$, 0.17 | | | |
| Lys-113 | $C_\alpha H$, 0.04 | | | | |
| Leu-118 | $C_\alpha H$, 0.06 | $C_\beta H$, 0.06 | $C_\delta H$, 0.07 | | |

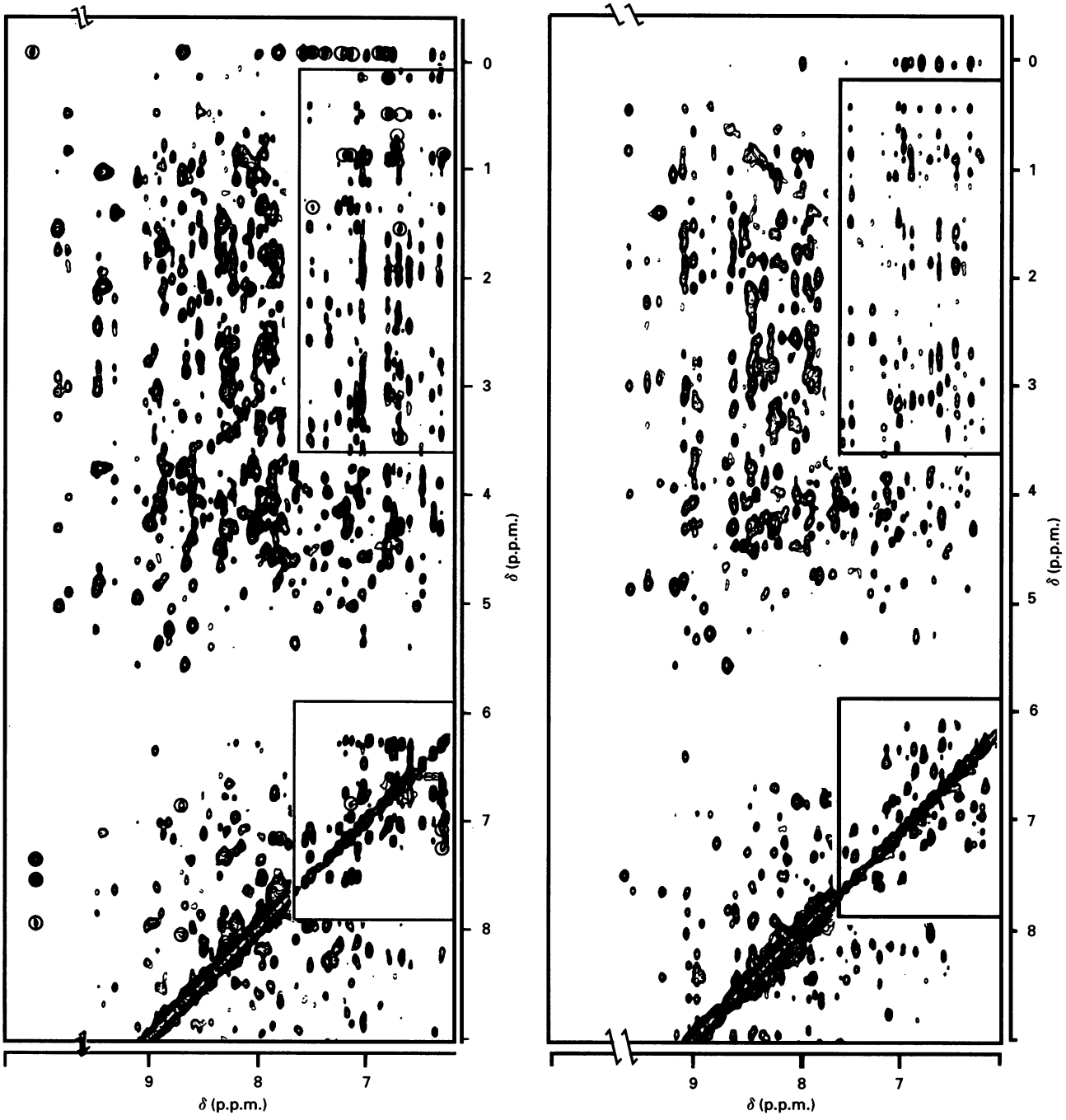


**Figure 5 NOESY spectra of $PLA_2$**

NOESY spectra ($\tau_m$ = 100 ms) of $PLA_2$ (2 mM) are shown: (**a**) in the presence of 50 mM SDS and no calcium; and (**b**) in the presence of 50 mM $CaCl_2$ and no SDS. The main spectra were acquired in $H_2O$; the boxed sections show spectra acquired in $^2H_2O$ to allow NOEs involving aromatic protons to be seen more easily. The increased NOEs in the presence of SDS are circled in (**a**). New or increased NOEs which are circled are from Ala-1 $C_\beta H_3$ (−0.07 p.p.m.) to Gln-4 NH (6.80 p.p.m.), Trp-3 NH (6.86 p.p.m.), Trp-3 $C_{\zeta 3}H$ (7.13 p.p.m.), Trp-3 $C_{\eta 2}H$ (7.19 p.p.m.), Trp-3 $C_{\delta 1}H$ (7.36 p.p.m.), Trp-3 $C_{\epsilon 3}H$ (7.49 p.p.m.), Trp-3 $C_{\zeta 2}H$ (7.56 p.p.m.), Ala-1 NH (7.79 p.p.m.), Leu-2 NH (8.70 p.p.m.), Trp-3 $N_{\epsilon 1}H$ (10.7 p.p.m.). From Leu-41 $C_\delta H_3$ (0.16 p.p.m.) to Tyr-111 $C_\epsilon H$ (6.78 p.p.m.). From Leu-41 $C_\delta H_3$ (0.50 p.p.m.) to Phe-106 $C_\epsilon H$ (6.74 p.p.m.), Tyr-111 $C_\epsilon H$ (6.78 p.p.m.). From Leu-118 $C_\delta H_3$ (0.72 p.p.m.) to His-115 $C_{\delta 2}H$ (6.75 p.p.m.). From Leu-118 $C_\delta H_3$ (0.80 p.p.m.) to His-115 $C_{\delta 2}H$ (6.75 p.p.m.). Ala-102 $C_\beta H_3$ (0.88 p.p.m.) to Phe-5 $C_\zeta H$ (6.26 p.p.m.), Phe-5 $C_\delta H$ (7.12 p.p.m.) and Phe-106 $C_\zeta H$ (7.18 p.p.m.). Leu-2 $C_\delta H_3$ (1.36 p.p.m.) to Trp-3 $C_{\epsilon 3}H$ (7.49 p.p.m.). Ala-5 $C_\beta H$ (1.55 p.p.m.) to Tyr-52 $C_\delta H$ (6.66 p.p.m.). From Ala-55 $C_\alpha H$ (3.48 p.p.m.) to Tyr-52 $C_\delta H$ (6.66 p.p.m.). From His-48 $C_{\delta 2}H$ (6.85 p.p.m.) to Phe-5 $C_\delta H$ (7.12 p.p.m.). From Trp-3 NH (6.87 p.p.m.) to Leu-2 NH (8.70 p.p.m.). From Tyr-52 $C_\epsilon H$ (6.28 p.p.m.) to Tyr-69 $C_\epsilon H$ (7.06 p.p.m.) and to Tyr-69 $C_\delta H$ (7.24 p.p.m.). From Trp-3 $C_{\eta 2}H$ (7.19 p.p.m.) to Ala-1 NH (7.79 p.p.m.). From Trp-3 $N_{\epsilon 1}H$ (10.74 p.p.m.) to Gln-4 NH (7.96 p.p.m.), to Trp-3 $C_{\zeta 2}H$ (7.56 p.p.m.), Trp-3 $C_{\delta 1}H$ (7.36 p.p.m.) and Trp-3 $C_{\eta 2}H$ (7.19 p.p.m.). From Phe-5 NH (8.05 p.p.m.) to Leu-2 NH (8.70 p.p.m.).

**Table 2 Changes in NOEs observed upon addition of SDS to $PLA_2$**

NOE intensities were estimated as peak heights from the spectra shown in Figure 5 and comparable spectra recorded in $^2H_2O$, and classified into strong (s), medium (m) and weak (w) cross-peaks; – indicates that the cross-peak was unobservable. Chemical shifts of these cross-peaks are listed in the legend to Figure 5.

| | | NOE intensity in the presence of | |
|---|---|---|---|
| Proton | Proton | $Ca^{2+}$ | SDS |
| Ala-1 NH | Leu-2 NH | – | w |
| Ala-1 NH | Trp-3 $N_{\varepsilon 2}H$ | – | w |
| Ala-1 NH | Gln-4 $C_\beta H$ | – | s |
| Ala-1 $C_\alpha H$ | Leu-2 NH | – | s |
| Ala-1 $C_\beta H_3$ | Leu-2 NH | – | s |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_{\zeta 3}H$ | – | s |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_{\eta 2}H$ | – | s |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_{\delta 1}H$ | – | s |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_{\varepsilon 3}H$ | – | m |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_{\zeta 2}H$ | – | s |
| Ala-1 $C_\beta H_3$ | Trp-3 $C_\alpha H$ | – | m |
| Ala-1 $C_\beta H_3$ | Gln-4 NH | – | s |
| Leu-2 NH | Trp-3 NH | – | m |
| Leu-2 NH | Phe-5 NH | – | m |
| Leu-2 $C_{\delta 1}H_3$ | Trp-3 $C_{\zeta 2}H$ | – | m |
| Phe-5 $C_\delta H$ | His-48 $C_{\delta 2}H$ | – | s |
| Phe-5 $C_\delta H$ | Ala-102 $C_\beta H_3$ | – | s |
| Phe-5 $C_\zeta H$ | Ala-102 $C_\beta H_3$ | – | s |
| Val-38 $C_{\gamma 1}H_3$ | Leu-118 $C_{\delta 2}H_3$ | – | s |
| Val-38 $C_{\gamma 1}H_3$ | Leu-118 $C_{\delta 1}H_3$ | – | s |
| Val-38 $C_{\gamma 1}H_3$ | His-115 $C_{\delta 2}H$ | s | m |
| Leu-41 $C_{\delta 2}H_3$ | Tyr-111 $C_\varepsilon H$ | – | m |
| His-48 $C_{\delta 2}H$ | Ala-102 $C_\beta H_3$ | s | – |
| His-48 $C_{\delta 2}H$ | Tyr-111 $C_\varepsilon H$ | – | m |
| Tyr-52 $C_\delta H$ | Ala-55 $C_\alpha H$ | – | s |
| Tyr-52 $C_\delta H$ | Ala-55 $C_\beta H_3$ | m | s |
| Tyr-52 $C_\varepsilon H$ | Tyr-69 $C_\delta H$ | w | s |
| Tyr-52 $C_\varepsilon H$ | Tyr-69 $C_\varepsilon H$ | w | s |
| Ala-102 $C_\beta H_3$ | Phe-106 $C_\zeta H$ | – | s |
| His-115 $C_{\delta 2}H$ | Leu-118 $C_{\delta 1}H_3$ | – | s |
| His-115 $C_{\delta 2}H$ | Leu-118 $C_{\delta 2}H_3$ | – | s |

addition of SDS. As for the chemical shift changes, there are prominent changes in residues near the N-terminus of the protein, with a number of NOEs involving residues 1–5 being observed only in the presence of SDS. Other residues which show changed NOEs are predominantly in or near the active-site cleft (residues 5, 48, 52, 69, 98, 99 and 102); the only exceptions to this are the appearance of NOEs between Val-38 and Leu-118, Leu-41 and Tyr-111, and His-115 and Leu-118. The relevant cross-peaks are shown ringed in Figure 5, and the changes are listed in Table 2.

### Active site versus micelle binding

If SDS is indeed a substrate analogue, it is likely to bind at the active site, and indeed some of the changes seen in the n.m.r. spectrum on SDS binding, such as those involving Tyr-52 and Tyr-69, are characteristically seen when substrate analogues bind to the active site [24]. It is important to establish whether the marked effects of SDS-binding on the N-terminal residues are a consequence of SDS binding in the active site or elsewhere on the molecule. In order to make this distinction, we examined SDS binding to $PLA_2$ which had been chemically modified on the active site His-48. Reaction of $PLA_2$ with *p*-bromophenacylbromide is known to result in stoichiometric reaction with His-48, resulting in inactivation of the enzyme by blocking the active site [25]. In the n.m.r. spectrum of the modified enzyme, an NOE was observed between the aromatic protons of Tyr-52 and those of the *p*-bromophenacyl group, but the changes involving the N-terminal residues seen after SDS addition were not seen and, as for the unmodified enzyme, the Ala-1 methyl resonance was not observable. However, upon addition of 50 mM SDS to the modified enzyme, the NOE changes observed for the N-terminal residues were very similar to those observed in unmodified $PLA_2$. In particular NOEs appeared between Ala-1 methyl and Trp-3 aromatic protons, and between Leu-2 methyl and Phe-5 aromatic protons.

This experiment showed that the changes at the N-terminus of $PLA_2$ on addition of SDS were not induced by the presence of a *p*-bromophenacyl group in the active site but that they could still be induced by the addition of SDS. To investigate the effects of the presence of a long alkyl chain in the active site, we have synthesized two long-chain α-bromoketones and used them to modify $PLA_2$. Both 1-bromoheptadecan-2-one ($C_{17}$-ABK) and 1-bromononadecan-2-one ($C_{19}$-ABK) reacted slowly with $PLA_2$ to yield catalytically inactive enzyme. The mass difference, measured by electrospray m.s., between unmodified $PLA_2$ and $PLA_2$ modified with $C_{19}$-ABK ($C_{19}$-ABK-$PLA_2$) was 279.4 ($\pm 1.36$), demonstrating that the reaction proceeded with 1:1 stoichiometry (for which the calculated mass difference is 280). The site of modification was assumed on chemical grounds to be His-48, although this was not demonstrated directly.

The n.m.r. spectra of $PLA_2$ modified with α-bromoketones were assigned by analogy to the spectra of the unmodified enzyme. The spectra of the two modified enzymes are generally very similar. The most notable effect of the modification is the shift of the *meta* proton resonance of Tyr-52 from 6.30 p.p.m. to 5.70 p.p.m, and in addition it was not possible, in either case, to locate the $H_\gamma$-$C_\delta H_3$ crosspeaks of Leu-2. We expect that this is due to resonance broadening, which suggests a slow conformational change in this region of the protein [26]. The main differences between spectra of $C_{19}$-ABK-$PLA_2$ and the $C_{17}$-ABK-$PLA_2$ proteins were seen in the chemical shifts of the resonances of Trp-3, whose aromatic resonances were more dispersed in spectra of the $C_{19}$-ABK-modified enzyme than in the $C_{17}$ derivative, and Tyr-69, whose *ortho* protons differed in shift by 0.2 p.p.m. Importantly, in both cases the general pattern of NOEs involving Trp-3 was similar to that seen in the unmodified protein, and in neither modified protein were NOEs between Trp-3 and the Ala-1 methyl protons observable. However, the addition of 50 mM [$^2H_{25}$]SDS to the sample of $C_{17}$-ABK-$PLA_2$ produced a set of NOE changes corresponding closely to those seen with the unmodified protein. Specifically, the methyl resonance of Ala-1 was observed at high field and gave clear NOEs to the Trp-3 aromatic protons.

## DISCUSSION

The results described in this paper show that bovine $PLA_2$, an enzyme which very efficiently hydrolyses anionic phospholipids in micellar form, is not denatured by SDS at concentrations up to 50 mM, but rather forms a micellar complex which may serve as a simple model system in which to study the structural changes in the enzyme which accompany binding to a micelle. Binding curves measured by both fluorescence, which reports on the local environment of Trp-3, and by microcalorimetry, which detects any interaction associated with a significant heat release, are complex. The fluorescence binding-curve involved three phases, which are not yet fully understood but which might be interpreted in terms of SDS binding to the active site, formation of a micellar $PLA_2$–SDS complex and a change in the structure of the complex at higher SDS concentrations. Other workers have studied

changes in fluorescence for interactions of $PLA_2$ with lipid analogues and shown the fluorescence changes to originate from $PLA_2$-micelle binding and then binding of an inhibitor to the active site of the enzyme while the enzyme is still bound to the micelle [27]. These fluorescence changes all contribute to the titration curve. If these processes have different equilibrium constants then this could account for the multi-phase fluorescence titration curves we have observed. N.m.r. spectroscopy provides strong, though indirect, evidence that SDS does indeed bind to the active site of $PLA_2$, but also shows that the interaction with SDS at higher concentrations produces changes in resonances from residues elsewhere in the molecule.

Equilibrium dialysis and ultracentrifugation allow quantitative determination of the composition of the $PLA_2$–SDS complexes formed at SDS concentrations which produce these additional changes in the n.m.r. spectrum. Equilibrium dialysis allows the determination of the amount of SDS bound per $PLA_2$ molecule, but cannot determine the size of the individual complexes. Ultracentrifugation enables the molecular mass of the protein component of the complex to be measured, regardless of the amount of SDS in the complex or the partial specific volumes of the protein and SDS when present in the complex. The value of 17.6 ($\pm$2.4) kDa obtained shows that more than one $PLA_2$ is present in some of the complexes. However, the plots of protein concentration against distance down the ultracentrifuge cell (not shown) gave no evidence for polydispersity, implying that there is a rapid equilibrium between species with one $PLA_2$ and those with more than one protein monomer in the complex. The ultracentrifugation data allow only a very imprecise estimate of the partial specific-volume of the complex and hence the number of SDS molecules in the complex. However a combination of the ultracentrifuge and equilibrium dialysis data does allow the characterization of the average species present in the sample as containing 26.4 ($\pm$3.1) molecules of SDS and 1.28 ($\pm$0.17) protein molecules.

In the n.m.r. spectra, chemical shift changes on addition of 50 mM SDS have been observed for 58 residues of $PLA_2$ (Table 1), and for a more limited number of residues there are also clear changes in NOE intensities (Table 2); with very few exceptions, the residues showing the largest changes in chemical shift also show changes in NOEs. Previous work using porcine $PLA_2$ reported a number of changes in chemical shifts and NOEs on binding of the enzyme to dodecylphosphocholine micelles [28,29]. Of the changes in chemical shift reported here for the bovine $PLA_2$–SDS complex, approximately 30% can be compared with the more limited data for the porcine $PLA_2$–dodecylphosphocholine complex. Most are strikingly similar in direction and magnitude, only 7 out of 53 differing by more than 0.1 p.p.m.; specific comparisons are made below.

Two residues which show significant changes in shift on SDS binding are Tyr-28 and Cys-29, which are both close to the calcium-binding site. It is important to note that in the comparison of n.m.r. spectra, the sample without SDS contained 50 mM $CaCl_2$, required to maintain the stability of the protein at the temperature of the n.m.r. experiment, while the sample containing SDS contained no calcium (to avoid precipitation of the calcium salt of SDS). The chemical shift changes in Tyr-28 on adding SDS are very similar to those observed in the absence of calcium from the sample. The chemical shifts of Tyr-28 NH, $C_\delta H$ and $C_\epsilon H$ in calcium-free $PLA_2$ (at somewhat lower temperature) are all within 0.04 p.p.m. of the shifts in the presence of SDS. We conclude, therefore, that the chemical shift changes in the signals of this residue in the calcium-binding site must be due to the absence of calcium rather than the presence of SDS.

The remaining residues affected by SDS binding can be divided into three groups, on the basis of their location in the protein. First, SDS clearly affects both chemical shifts and NOEs of a number of residues which are in or near the active site. These include His-48 (the catalytically essential histidine residue), Phe-5, Tyr-52, Tyr-69, Cys-98, Asp-99 and Ala-102. These changes, in both shift and NOEs, are all very similar to those observed on the binding of a single substrate analogue into the active site [24,30], strongly suggesting that they result from the binding of a single SDS to the active site as a substrate analogue. In this region, only a small number of changes have been observed for signals assigned in both the bovine and porcine enzymes; the chemical shift changes in Tyr-69 ($H_\epsilon$ 0.26 versus 0.17, $H_\delta$ 0.06 versus 0.10 p.p.m; bovine versus porcine) and Cys-98 (NH $-0.27$ versus $-0.15$ p.p.m.) are clearly different.

The largest changes in the n.m.r. spectrum upon the addition of SDS involve residues near the N-terminus. The resonances of Ala-1, which are not detectable in the spectrum of the protein alone, are clearly visible in the SDS complex, the Leu-2 $H_\gamma$ and Trp-3 H7 resonances show chemical shift changes of $> 0.5$ p.p.m, and there are a number of new NOESY cross-peaks involving residues 2–5. The N-terminal helix was noted to be somewhat disordered in the crystal structure of the enzyme alone [31], and this might lead to line-broadening of the resonances of Ala-1 sufficient to make them undetectable in the absence of SDS. The appearance of the Ala-1 resonances on addition of SDS indicates that this N-terminal part of the structure is more ordered in the micellar complex than in the protein alone. The fact that the $^1H$ resonance of the terminal $-NH_3^+$ (7.79 p.p.m.) group is readily observable in the spectrum of the complex at pH 6.1 is particularly striking and must indicate that this group is significantly more buried and/or more strongly hydrogen-bonded in the complex than in the free protein. The chemical shift of the Ala-1 methyl resonance is substantially upfield from its random coil value, presumably due to the magnetic anisotropy of Trp-3, and there are six NOEs from Ala-1 methyl protons to protons of Trp-3. Both these observations indicate that the relative position of these two side-chains is well-defined in the complex. Further evidence for increased order at the N-terminus comes from the appearance of a number of new NOEs involving residues in this region. These include several new sequential NOEs, namely Ala-$1_{NH}$–Leu-$2_{NH}$, Leu-$2_{NH}$–Trp-$3_{NH}$, Ala-$1_{NH}$–Gln-$4_{H\beta}$ and Ala-$1_{H\beta}$–Gln-$4_{NH}$, strongly suggesting that the helical structure of this part of the molecule extends all the way to the N-terminus in the SDS complex.

Of the first five residues, information is available from the porcine $PLA_2$–dodecylphosphocholine micellar complex [26,27] for Trp-3, Gln-4 and Phe-5. Resonances of these residues all show changes in chemical shift on micelle binding, but several of these differ in magnitude from those reported here for the bovine $PLA_2$–SDS complex. For example, for Trp-3, the following shift changes (in p.p.m.) are seen for the bovine and porcine enzymes respectively: H5, $-0.15$ versus $-0.15$; H6, $-0.12$ versus $-0.13$, but H2, $-0.15$ versus $-0.01$; H4, $-0.12$ versus $-0.03$; H7, $-0.06$ versus 0.04; indole NH, 0.52 versus 0.90. It appears from this comparison that there are differences in the chemical shift changes for each enzyme. The differences in chemical shift observed between the two micelle types may simply reflect the different chemical environment provided by the SDS molecules. However, the NOE changes in this region for each of the two complexes are not identical, implying that the structural change might not be the same.

The binding of several short or medium chain-length inhibitors to the active site in 1:1 stoichiometry has been found to produce characteristic changes in chemical shifts and NOEs for residues in the active site (see above) but not in the N-terminal residues

[24,32]. However, we have observed effects on the N-terminal residues very similar to those reported here for SDS in a 1:1 complex of $PLA_2$ with an inhibitor, 3-thio-octadecanyl-2-(R)-acetylaminophosphatidylcholine (RLM-1), which has a $C_{18}$ alkyl chain at the $C_1$ position (W. U. Primrose and R. L. Magolda, unpublished work). The formation of the 1:1 complex with RLM-1 can be followed by n.m.r. and this shows NOE changes very similar to other ligands which produce 1:1 complexes [32]. The observation that SDS binding to $PLA_2$ inhibited by *p*-bromophenacyl bromide still produced the characteristic changes at the N-terminus demonstrated that these effects are not due to SDS binding at the active site. In order to explore further whether simple occupation of the active site by a long alkyl chain is sufficient to change the conformation of the N-terminal part of the molecule, we prepared and studied two derivatives of $PLA_2$ having long alkyl chains covalently attached in the active site, almost certainly to the active-site histidine residue. It was clear from the n.m.r. spectra that the change in structure at the N-terminus had not taken place in these modified enzymes, although it could be induced by subsequent addition of SDS. There is thus a difference between these observations and those with the non-covalent inhibitor RLM-1. It is possible that the alkyl chain of the α-bromoketones might not have been quite long enough to produce the effect; in RLM-1 there are 24 atoms between the histidine residue and the end of the alkyl chain whereas in the longer of the two covalent inhibitors there are only 20. Alternatively, it is possible that the alkyl chain in the α-bromoketone-modified enzyme does not bind in the same way as the alkyl chain of RLM-1; further work will be required to distinguish between these possibilities.

The final group of residues affected by SDS binding are Tyr-111, Lys-113, His-115 and Leu-118; these residues are not close to either the active site (with the exception of Tyr-111) or the N-terminus. Some chemical shift changes are seen for these residues in the porcine $PLA_2$–dodecylphosphocholine complex [26,27], notably for Tyr-111, but the additional NOEs involving Tyr-111, His-115 and Leu-118 seen here were not reported for the porcine enzyme complex. The origin of the changes involving these residues is more difficult to explain, although it is interesting to note that this C-terminal region of the protein contains four lysines (113, 116, 121 and 122), forming a positively charged 'patch' to which one might expect negatively charged SDS molecules to bind. It remains to be established whether this putative interaction contributes to the activation of the enzyme.

A good deal of earlier evidence, to some extent indirect, has implicated the N-terminal residues of $PLA_2$ in the activation by binding to aggregated substrate [33,34]. The present work provides direct evidence for a change in conformation of this part of the protein molecule when $PLA_2$ binds to SDS micelles, specifically for increased order and increased helical structure in the first five residues.

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## REFERENCES

1 Achari, A., Scott, D., Barlow, P., Vidal, J. C., Otwinowski, Z., Brunie, S. and Sigler, P. B. (1987) Cold Spring Harbour Symp. Quant. Biol. **52**, 441–452
2 Verheij, H. M., Slotboom, A. J. and de Haas, G. H. (1981) Rev. Physiol. Biochem. Pharmacol. **9**, 91–203
3 Chang, J., Musser, J. H. and McGregor, H. (1987) Biochem. Pharmacol. **36**, 2429–2436
4 Waite, M. (1987) Handbook of Lipid Research, vol. 5, Plenum Press, New York
5 Burgoyne, R. D. and Morgan, A. (1990) Trends Biochem. Sci. **15**, 365–366
6 Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. and Sigler, P. B. (1990) Science **250**, 1541–1546
7 Thunnissen, M. M. G. M., Ab, E., Kalk, K. H., Drenth, J., Dijkstra, B. W., Kuipers, O. P., Dijkman, R., de Haas, G. H., Verheij, H. M. (1990) Nature (London) **347**, 689–691
8 Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J. and Sigler, P. B. (1985) J. Biol. Chem. **260**, 11627–11634
9 Kuipers, O. P., Vincent, M., Brochon, J.-C., Verheij, H. M., de Haas, G. H. and Gallay, J. (1992) Biochemistry **30**, 8771–8785
10 Peters, A. R., Dekker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A. J. and De Haas, G. H. (1992) Biochemistry **31**, 10024–10030
11 Jain, M. K., Ranadive, G., Yu, B.-Z. and Verheij, H. M. (1991) Biochemistry **30**, 7330–7340
12 Dutilh, C. E., Van Doren, P. J., Verheul, F. E. A. M. and de Haas, G. H. (1975) Eur. J. Biochem. **53**, 91–97
13 SAS Institute Ins., SAS Circle, P.O. Box 8000, Cary, N.C. 27512-8000, U.S.A.
14 De Haas, G. H., Postema, N. M., Nieuwenhuizen, W. and Van Deenen, L. L. M. (1968) Biochim. Biophys. Acta **159**, 103–117
15 Plateau, P. and Guéron, M. (1982) J. Am. Chem. Soc. **104**, 7310–7311
16 Marion, D. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. **113**, 967–974
17 Wider, G., Maura, S., Kumar, A., Ernst, R. R. and Wutrich, K. (1984) J. Magn. Reson. **56**, 207–234
18 Williamson, M. P., Marion, D. and Wuthrich, K. (1984) J. Mol. Biol. **173**, 341–359
19 Bax, A. and Davis, D. G. (1985) J. Magn. Reson. **65**, 355–360
20 Bothner-By, A. A., Stephens, R. L., Lee, J.-M., Warren, C. D. and Jeanloz, R. W. (1984) J. Am. Chem. Soc. **106**, 811–813
21 Reynolds, J. A. and Tanford, C. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 4467–4470
22 Ikeda, K., Sano, S., Teshima, K. and Samejima, Y. (1984) J. Biochem. **96**, 1427–1436
23 Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R., Dijkman, R. and de Haas, G. H. (1982) Biochim. Biophys. Acta **688**, 341–348
24 Bennion, C., Connolly, S., Gensmantel, N. P., Hallam, C., Jackson, C. G., Primrose, W. U., Roberts, G. C. K., Robinson, D. H. and Slaich, P. K. (1992) J. Med. Chem. **35**, 2939–2951
25 Volwerk, J. J., Pieterson, W. A. and de Haas, G. H. (1974) Biochemistry **13**, 1446–1454
26 Fisher, J., Primrose, W. U., Roberts, G. C. K., Dekker, N., Boelens, R., Kaptein, R. and Slotboom, A. J. (1989) Biochemistry **28**, 5939–5946
27 Jain, M. K. and Maliwal, B. P. (1993) Biochemistry **32**, 11838–11846
28 Peters, A. R., Dekker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A. J. and De Haas, G. H. (1992) Biochemistry **31**, 10024–10030
29 Dekker, N., Peters, A. R., Slothboom, A. J., Boelens, R., Kaptein, R., Dijkman, R. and de Haas, G. H. (1991) Eur. J. Biochem. **199**, 601–607
30 Peters, A. R., Dekker, N., Van den Berg, L., Boelens, R., Slotboom, A. J., de Haas, G. H. and Kaptein, R. (1992) Biochimie **74** 859–866
31 Dijkstra, B. W., Kalk, K. H., Hol. W. G. J. and Drenth, J. (1981) J. Mol. Biol. **147**, 97–123
32 Slaich, P. K., Primrose, W. U., Robinson, D. H., Wharton, C. W., White, A. J., Drabble, K. and Roberts, G. C. K. (1992) Biochem. J. **288**, 167–173
33 Slothboom, A. J. and De Haas, G. H. (1975) Biochemistry **14**, 5394–5399
34 Kuipers, O. P., Vincent, M., Brochon, J.-C., Verheij, H. M., De Haas, G. H. and Gallay, J. (1991) Biochemistry **30**, 8771–8785