^{1}H NMR Studies of Bovine and Porcine Phospholipase A_2: Assignment of Aromatic Resonances and Evidence for a Conformational Equilibrium in Solution†

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ABSTRACT: Bovine and porcine pancreatic phospholipases A_2, and porcine isophospholipase A_2, have been investigated by one- and two-dimensional ^{1}H NMR spectroscopy. Resonances have been assigned for 20–26 residues in each enzyme, including all the aromatic residues, by a strategy based on the semiquantitative comparison of proximity relationships deduced from NOE experiments with those seen in the crystal structure. NOE experiments indicate that the loop comprising residues 59–70, which has a different conformation in the crystal structures of the bovine and porcine enzymes, has the same conformation in these two enzymes in solution. Selective changes in the line width of a limited number of resonances as a function of pH, temperature, and calcium concentration provide evidence for a local conformational equilibrium. This equilibrium involves a limited region of the protein structure around residues 25, 41, 106, and 111; it has been identified in the bovine enzyme and porcine isoenzyme but is not apparent in the porcine enzyme.

Phospholipase A_2 (PLA_2,[1] EC 3.1.1.4) is a small calcium-dependent enzyme that catalyzes the hydrolysis of the 2-acyl ester bond of phospholipids to release a fatty acid, notably arachidonic acid, which is, in turn, the precursor of the eicosanoids, including the prostaglandins, thromboxanes, and leukotrienes (Verheij et al., 1981; Achari et al., 1987; Chang et al., 1987; Waite, 1987). These have in common a role as mediators of inflammatory and acute allergic responses. While tissue PLA_2 has not yet been purified in sufficient quantities for detailed study, the pancreatic enzyme is readily available and has been extensively studied [Nieuwenhuizen et al., 1974; Dutilh et al., 1975; Verheij et al. (1981) and references cited therein], and there is good evidence that it is very similar to the tissue enzyme, in terms of both amino acid sequence (Forst et al., 1986) and immunological cross-reactivity (Okamoto et al., 1985). The three-dimensional structure of the pancreatic enzyme has been determined by X-ray crystallography (Dijkstra et al., 1981, 1983; Renetseder et al., 1988), although as yet no crystal structure of the enzyme with a substrate analogue bound is available. We have embarked on a study of pancreatic phospholipase A_2 by two-dimensional NMR at 500 MHz, with the aim of understanding the binding of substrate analogues and inhibitors and thereby contributing to the design of improved inhibitors, which may have potential as antiinflammatory agents. There have been a number of earlier studies of the enzyme by NMR at lower fields (Aguiar, 1979; Aguiar et al., 1979; Jansen et al., 1978, 1979; Egmond et al., 1980, 1983), but as yet only a limited number of resonance assignments have been made in the ^{1}H NMR spectrum of the protein. Such assignments are an essential first step in a detailed NMR study, and in this paper we report assignments for the aromatic amino acid residues of the bovine and porcine enzymes and the porcine isoenzyme. In addition, we describe evidence for the existence of a conformational equilibrium in a localized region of the protein structure.

Materials and Methods

Bovine and porcine PLA_2 and porcine iso-PLA_2 were prepared from pancreas following literature procedures (Nieuwenhuizen et al., 1974; Dutilh et al., 1975; van Wezel & de Haas, 1975). Phe^5-$^2H_5$-labeled ϵ-amidinated bovine PLA_2 (AMPA) was prepared as previously described (Allegrini et al., 1985). Samples for the NMR investigations were prepared in two ways. In the first, the enzyme was dissolved in 99.99% ^2H_2O at pH* 4.1 and then lyophilized, the dissolution/lyophilization procedure being repeated at least five times. Alternatively, to replace all exchangeable protons by deuterons, the enzyme was dissolved in 99.99% ^2H_2O (Aldrich Chemical Co.) at pH* 2–3 and the solution heated to 70 °C for 10 min, followed by repeated lyophilization and dissolution in ^2H_2O. The NMR samples were typically 1.0–1.5 mM enzyme in 0.4 mL of ^2H_2O, either containing 50 mM $CaCl_2$ and 200 mM NaCl or containing 300 mM NaCl. The pH* was adjusted by the addition of microliter quantities of ^2HCl and NaO^2H (Aldrich Chemical Co.). pH* denotes a meter reading, uncorrected for the deuterium isotope effect on the glass electrode. Calcium ion titrations at constant ionic strength were carried out by adding an enzyme solution containing 150 mM

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[1] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; PLA_2, phospholipase A_2; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; COSY, correlated spectroscopy; NOESY, 2-D nuclear Overhauser effect or enhancement spectroscopy; NOE, nuclear Overhauser effect or enhancement; 1-D, one dimensional; 2-D, two dimensional; AMPA, ϵ-amidinated phospholipase A_2; ppm, parts per million; photo-CIDNP, photochemically induced dynamic nuclear polarization.

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$CaCl_2$ to the starting solution containing 300 mM NaCl, both solutions being at pH 7.1.

All ^1H NMR spectra were recorded at 500 MHz on a Bruker AM500. Chemical shifts were measured relative to internal dioxane and are reported relative to 2,2-dimethyl-2-silapentane 5-sulfonate (DSS), the chemical shift of dioxane being taken as 3.77 ppm from DSS.

The resonance of residual H_2O was suppressed in all cases by preirradiation of the water resonance for 1.0–1.5 s. Phase-sensitive 2-D spectra were recorded with the time proportional phase increment (TPPI) method (Marion & Wuthrich, 1983). 2-D spectra were acquired with 250–850 t_1 values in 1024 or 2048 data points. Prior to Fourier transformation, the data were zero filled (either in both dimensions or only in f_2) and multiplied by a window function (Gaussian, shifted sine bell or sine bell squared). The NOESY spectrum of the bovine enzyme was base line corrected after transformation with a fourth-order polynomial.

The HOHAHA (Bax & Davis, 1985; Davis & Bax, 1985) spectra were recorded in a spin locking field created by an MLEV-17 pulse train, with the decoupler channel used as transmitter and receiver.

Results and Discussion

The first step in the assignment procedure is to link resonances that are part of the same spin system (and hence from the same amino acid) and to identify the type of amino acid residue from which they arise. A combination of two-dimensional COSY and HOHAHA (Bax & Davis, 1985; Davis & Bax, 1985) spectra enables all the spin–spin connectivities of an individual spin system to be identified. We have found the relayed coherence transfer experiment (Bax & Drobny, 1985; Weber et al., 1985a,b) to be uniformative in this system, presumably due to the short T_2 values expected for a molecule of this size. The identified spin systems are then assigned to specific amino acid residues in the sequence by comparison of connectivities observed in NOESY spectra in solution with spatial relationships observed by X-ray diffraction in the crystal, a strategy described earlier for dihydrofolate reductase (Hammond et al., 1986). In addition, in one case, the use of enzyme deuterated specifically in a single amino acid residue provides an unambiguous assignment. This strategy for making sequence-specific resonance assignments is obviously based on the assumption that the crystal and solution structures are nearly the same. However, we make only semiquantitative use of structural information for assignments; the observation of an NOE between two protons is taken to mean only that they are within 5 Å in the structure, and assignments are made only where this upper limit suffices for an unambiguous identification of the protons involved. An exact identity of the structures in solution and in the crystal need not, therefore, be assumed, and minor differences will not invalidate the assignments. To date, the limited data available are consistent with the hypothesis that the crystal and solution structures of phospholipase A_2 are the same; as the number of assignments increases, so more stringent tests of this will become possible.

The aromatic regions of the 500-MHz ^1H NMR spectra of bovine and porcine PLA_2 and porcine iso-PLA_2 are shown in Figure 1. Each enzyme, but particularly porcine PLA_2, tends to aggregate at high concentrations, especially at neutral pH. The best-resolved NMR spectra have consequently been obtained at concentrations of <1.5 mM and pH* 4–5. Note that the experimental conditions used for each enzyme differ slightly. The conditions used in each case represent the optimum for that particular protein. As can be seen from Figure 1, the spectra are well enough resolved for a number of individual resonances to be identified.

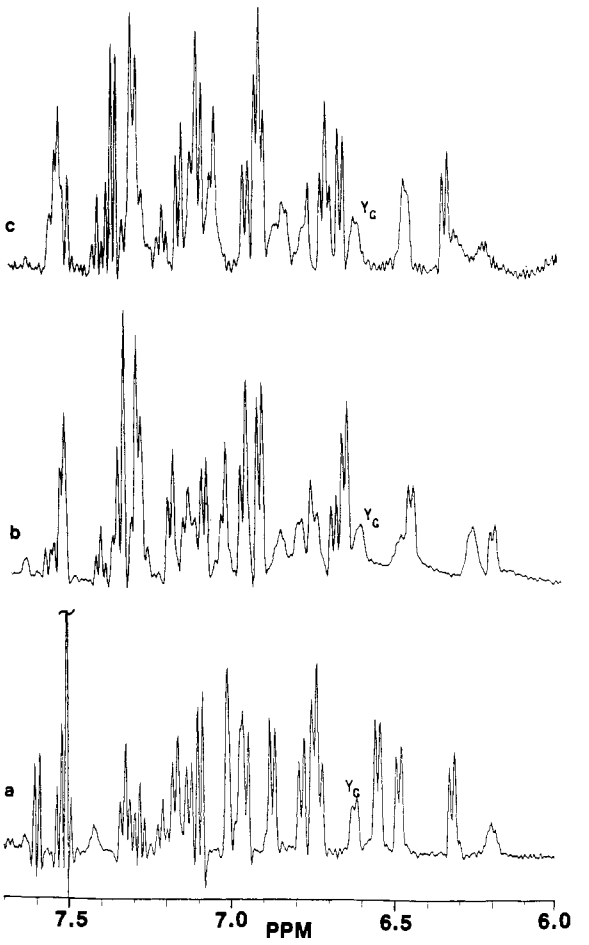

FIGURE 1: Aromatic region of the 500-MHz ^1H NMR spectra of three phospholipases A_2, in ^2H_2O solution at 320 K: (a) 1.5 mM bovine PLA_2 at pH* 5.2, 50 mM $CaCl_2$, and 200 mM NaCl; (b) 1 mM porcine PLA_2 at pH* 4.1, 50 mM $CaCl_2$, and 300 mM NaCl; (c) 1 mM porcine iso-PLA_2 at pH* 4.0, 50 mM $CaCl_2$, and 300 mM NaCl. The peak marked Y_G is a doublet resonance from Tyr-25 (see text).

Bovine PLA_2 has seven tyrosine, four phenylalanine, two histidine, and one tryptophan residues. The porcine enzyme and isoenzyme each have one more tyrosine (Tyr-123) and phenylalanine (Phe-63), while porcine PLA_2 also has an additional histidine (His-17). The aromatic region of the ^1H NMR spectrum of bovine PLA_2 is thus less complex than that of the others, and the line widths are also somewhat narrower, due to the lesser tendency of the bovine enzyme to aggregate. We shall therefore present briefly the evidence for resonance assignments for bovine PLA_2. A more detailed report of the assignment procedure is presented in the supplementary material, where reference is also made to the data for the porcine enzyme and isoenzyme. We shall see that the chemical shifts for the resonances of homologous residues are quite similar for the three enzymes, but similarities in NOE patterns, rather than chemical shifts, for homologous residues are used to transfer assignments from one enzyme to another.

Assignment of Aromatic and Associated ^1H Resonances. The spin systems of the aromatic rings of the unique tryptophan (W), three phenylalanines (F_A, F_B, and F_C) and six tyrosines (Y_A–Y_F) in the HOHAHA spectrum of bovine (Figure 2) and porcine PLA_2 (Figure 3) were identified by 1-D resonance multiplicities and intensities, HOHAHA cross-peak patterns, and the variation in cross-peak intensity with spin-lock mixing time. A seventh tyrosine (Y_H) and a fourth phenylalanine (F_E) spin system were additionally

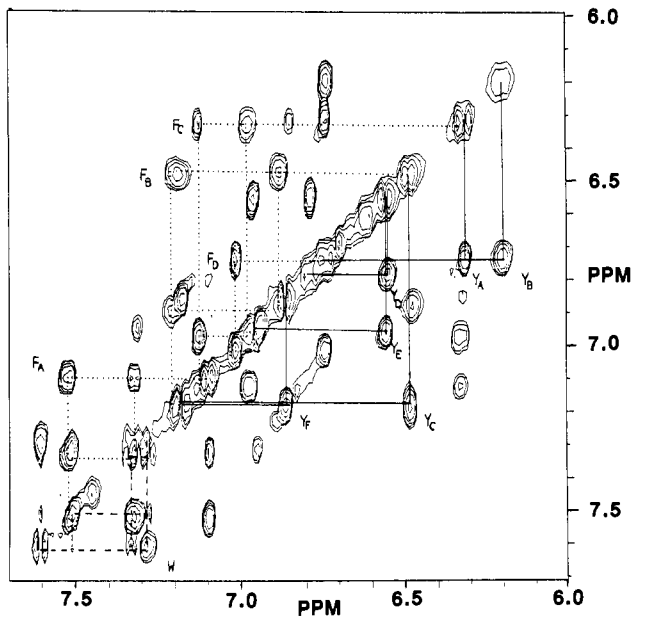

FIGURE 2: Aromatic region of the 500-MHz 2-D HOHAHA ^{1}H NMR spectrum of 1.5 mM bovine PLA_2 in ^2H_2O, 50 mM $CaCl_2$, and 200 mM NaCl, pH* 5.2, at 320 K. The spin systems of six of the seven tyrosines (Y_A–Y_F) and of the unique tryptophan (W) are shown below the diagonal and those of the four phenylalanines (F_A–F_D) above.

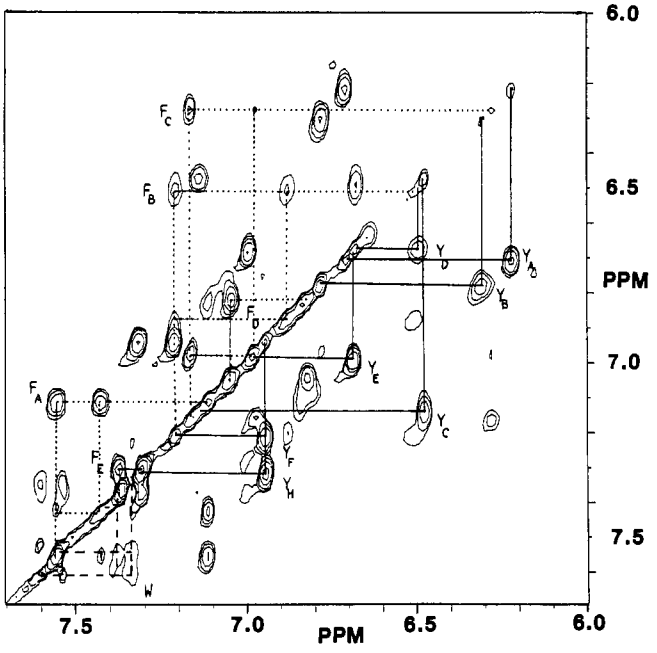

FIGURE 3: Aromatic region of the 500-MHz 2-D HOHAHA ^{1}H NMR spectrum of 1.0 mM porcine PLA_2 in ^2H_2O, 50 mM $CaCl_2$, and 300 mM NaCl, pH* 4.1, at 320 K. The spin systems of seven of the eight tyrosines (Y_A–Y_F and Y_H) and of the unique tryptophan (W) are shown below the diagonal and those of the five phenylalanines (F_A–F_E) above.

identified in the porcine spectrum with the same methods. All of these residues are ring flipping freely and give rise to first-order spin systems. The remaining phenylalanine spin system (F_D) was identified in spectra of both enzymes as a second-order system containing an intense signal with the appearance of a singlet at 7.0 ppm (see Figure 1). One resonance of the remaining tyrosine in each enzyme can be observed as a clear two-proton doublet in the 1-D spectra (indicated as Y_G in Figure 1). The resonance(s) of the remaining protons of this residue is (are) apparently broadened, presumably due to an exchange process, making them undetectable in both 1-D and 2-D spectra.

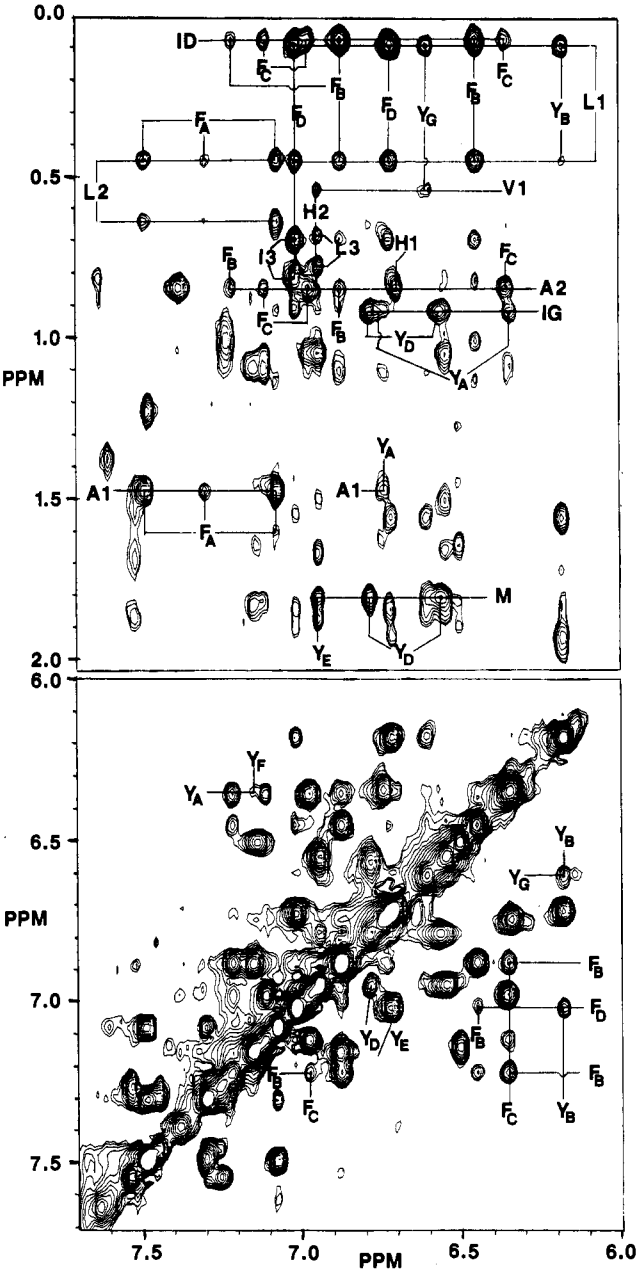

FIGURE 4: 500-MHz 2-D NOESY ^{1}H NMR spectrum of 2 mM bovine PLA_2, in ^2H_2O, 50 mM $CaCl_2$, and 200 mM NaCl, pH* 4.6, at 313 K. NOE connectivities between aromatic spin systems and other protons are indicated. Aromatic spin systems are labeled as in Figure 2. Other resonances are labeled as follows: H1 and H2, His C4H; L1, L2, and L3, Leu δ-methyl; V1, Val γ-methyl; A1 and A2, Ala β-methyl; ID, Ile δ-methyl; IG, Ile γ-methyl; I3, Ile γ,δ-methyls; M, Met S-methyl.

The histidine residues of the pancreatic PLA_2's have previously been assigned (Aguiar, 1979; Aguiar et al., 1979). We have examined the pH titration behavior of the C2H resonances and assigned the corresponding C4H resonances from the weak J coupling observed in the HOHAHA spectra (data not shown). However, the C2H resonance of the active site histidine, His-48, remains unassigned at neutral pH and temperatures below 70 °C.

Once spin systems were assigned to residue type, sequence-specific assignments were then made by observation of NOESY connectivities between aromatic systems and from aromatic systems to assignable methyl signals (Figure 4). From these, networks of spin systems were built up (Figure 5), which led to the sequence-specific assignment of the res-

Table I: ^{1}H Resonance Assignments[a] for Bovine and Porcine PLA_2 and Porcine Iso-PLA_2

		bovine PLA_2	porcine PLA_2	porcine iso-PLA_2			bovine PLA_2	porcine PLA_2	porcine iso-PLA_2
Trp-3	C4H	7.62	7.60	7.59	Tyr-75 (Y_E)	C_oH_2	6.95	6.98	6.99
	C5H	7.28	7.37	7.38		C_mH_2	6.55	6.68	6.67
	C6H	7.33	7.33	7.34		$C_\beta H_2$	3.12, 3.27	3.11, 3.18	3.15*
	C7H	7.52	7.54	7.53		$C_\alpha H$	5.34	5.44	5.44*
	C2H	7.51	7.54	7.52	Tyr-69 (Y_F)	C_oH_2	7.17	7.21	7.18
	$C_\beta H_2$	3.36, 3.51	3.42, 3.62	na		C_mH_2	6.87	6.95	6.94
	$C_\alpha H$	4.61	na	na		$C_\beta H_2$	3.08	na	na
	indole NH	10.29	10.20	na		$C_\alpha H$	4.50	na	na
Phe-94 (F_A)	C_oH_2	7.07	7.11	7.12	Tyr-25 (Y_G)	$C_\varepsilon H_2$	6.62	6.62	6.63
	C_mH_2	7.50	7.56	7.55	Tyr-123 (Y_H)	C_oH_2		7.30	7.29
	C_pH	7.30	7.43	7.42		C_mH_2		6.93	6.94
	$C_\beta H_2$	3.18, 3.34	3.19, 3.39	na		$C_\beta H_2$		3.20, 2.52	2.51, 3.21*
	$C_\alpha H$	4.02	4.02	na		$C_\alpha H$		4.64	4.63*
Phe-106 (F_B)	C_oH_2	6.36	6.51	6.56	Ile-9 (ID)	$C_\delta H_3$	0.04	0.02	0.07
	C_mH_2	6.83	6.86	6.88		$C_\gamma H_3$	1.19	1.12	1.21
	C_pH	7.20	7.20	7.24		$C_\gamma H$	0.17	0.19	0.24
	$C_\beta H_2$	2.62, 3.03	na	na	Ile-13 (I3)	$C_\delta H_3$	0.72	na	na
	$C_\alpha H$	4.25	na	na		$C_\gamma H_3$	0.84	na	na
Phe-5 (F_C)	C_oH_2	7.10	7.15	7.14	Val-38 (V1)	$C_\gamma H_3$	0.58, 0.89	0.55, 0.85	0.62, 0.90
	C_mH_2	6.98	6.96	6.93		$C_\beta H$	1.79	1.73	1.78
	C_pH	6.34	6.29	6.24	Leu-41 (L1)	$C_\delta H_3$	0.07, 0.47	0.11, 0.48	0.18, 0.48
	$C_\beta H_2$	3.19, 3.54	na	na		$C_\gamma H$	1.15	1.19	1.19
	$C_\alpha H$	4.38	4.51	na	Ala-55 (A1)	$C_\beta H_3$	1.50	1.53	1.55
Phe-22 (F_D)	C_oH_2	6.74	6.83	6.88		$C_\alpha H$	3.36	3.42	3.49
	C_mH_2	7.01	7.04	7.06	Leu-58 (L2)	$C_\delta H_3$	0.45, 0.65	0.45, 0.61	0.45, 0.64
	C_pH	7.01	7.04	7.06		$C_\gamma H$	1.55	1.51	1.55
	$C_\beta H_2$	na	3.31, 3.28	na		$C_\alpha H$	4.21	na	na
	$C_\alpha H$	na	4.33	na	Ser-74	$C_\beta H_2$	3.76, 3.84	3.76, 3.79	3.78
Phe-63 (F_E)	$C_\chi H$		~7.3	~7.3		$C_\alpha H$	5.26	5.21	5.22
Tyr-52 (Y_A)	C_oH_2	6.72	6.79	6.73	Cys-84	$C_\alpha H$	4.63	na	na
	C_mH_2	6.29	6.29	6.35	Ile-95 (IG)	$C_\gamma H_3$	0.92	0.94	na
	$C_\beta H_2$	2.80, 3.30	3.33, 3.0	na	Ala-102 (A2)	$C_\beta H_3$	0.89	na	na
	$C_\alpha H$	4.24	4.35	na		$C_\alpha H$	4.06	na	na
Tyr-111 (Y_B)	C_oH_2	6.72	6.70	6.80	Leu-118 (L3)	$C_\delta H_3$	0.68, 0.85	na	na
	C_mH_2	6.16	6.21	6.35		$C_\gamma H$	1.55	na	na
	$C_\beta H_2$	2.79, 2.99	3.11, 2.81	3.07, 2.75*		$C_\beta H_2$	1.31, 1.40	na	na
	$C_\alpha H$	4.28	4.30	4.24*		$C_\alpha H$	4.00	na	na
Tyr-28 (Y_C)	C_oH_2	6.46	6.46	6.48	His-17	C_2H		8.54	
	C_mH_2	7.17	7.13	7.13		C_4H		7.35	
Tyr-73 (Y_D)	C_oH_2	6.77	6.66	6.74	His-48 (H1)	C_2H	na	na	na
	C_mH_2	6.53	6.49	6.49		C_4H	6.72	na	na
	$C_\beta H_2$	2.76, 3.11	2.69, 3.12	2.72, 3.14*	His-115 (H2)	C_2H	8.23	8.78	na
	$C_\alpha H$	4.84	4.96	na		C_4H	6.99	6.83	na

[a] Chemical shifts expressed relative to DSS. Experimental conditions as follows: (bovine PLA_2) pH* 5.2, 200 mM NaCl, 50 mM $CaCl_2$, and 320 K; (porcine PLA_2) pH * 4.1, 300 mM NaCl, 50 mM $CaCl_2$, and 320 K; (porcine iso-PLA_2) pH* 4.0, 300 mM NaCl, 50 mM $CaCl_2$, and 320 K, except for those marked with an asterisk which were recorded at pH* 5.1. na, not assigned.

idues involved, after comparison with the three-dimensional structural information available from X-ray crystallography (Dijkstra et al., 1981).

An unequivocal assignment of the resonances of Phe-5 (to spin system F_C) was made by comparison of the 1-D and HOHAHA spectra of semisynthetic [*Phe*^{5}-$^2H_5$]AMPA (Allegrini et al., 1985) and isotopically normal AMPA. This was used as the starting point to identify all of the residues in the NOESY network involving F_C (top network in Figure 5), which contains 13 identifiable residue types connected by 19 observable NOE correlations between spin systems. Once around half of the aromatic residues were excluded by this method, almost all of the remainder could then be identified in the second NOE network (lower part of Figure 5), which contains 11 spin systems connected by 12 interresidue NOE's.

The remaining tyrosine (Y_C), for which no NOE correlation has been identified, was assigned to Tyr-28 by elimination, since the other six tyrosine residue in bovine PLA_2 exist as three pairs whose aromatic ring protons are in mutual proximity. The expected NOE's between three pairs of tyrosine spin systems can be observed in Figure 4.

All residues, for which at least one resonance has been assigned by this strategy, are listed in Table I. The detailed resonance assignment arguments and a comparison of the AMPA and [*Phe*^{5}-$^2H_5$]AMPA HOHAHA spectra are available as supplementary material for this paper.

We report assigned resonances for 26 out of 123 residues in bovine PLA_2 (23 in porcine PLA_2 and 20 in porcine iso-PLA_2), including all the aromatic residues (Table I). Many more assignments will be required for a detailed study of the behavior of the protein, and work aimed at a complete assignment of the spectrum is continuing; for example, resonance assignments for residues in three α-helices (A, C, and E) have been made (Dekker, Primrose, and Fisher, unpublished work) with the main chain directed strategy (Englander & Wand, 1987). Nonetheless, a good deal of useful information can be obtained from even the limited number of assignments reported here. In particular, assignments are now available for virtually all the hydrophobic residues which form the proposed substrate binding pocket, and studies of the mode of binding of substrate analogues and inhibitors are in progress (Primrose, Fisher, and Dekker, unpublished work).

Structural Comparison of the Three Enzymes Studied. In comparison with bovine PLA_2, there are 19 amino acid substitutions in the porcine enzyme, together with a single-residue insertion at position 120. A comparison of the crystal struc-

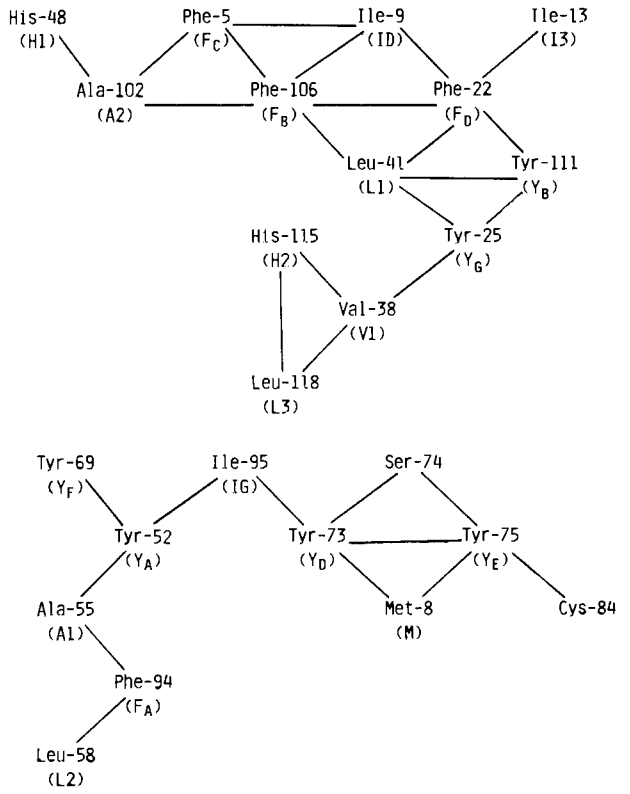

FIGURE 5: NOE networks identified in bovine PLA_2 from the data in Figure 4. Lines connect residues between which at least one NOE interaction has been observed. In each case these correspond to an interresidue proton–proton distance of <5 Å in the crystal structure. Spin system nomenclature used in the preceding figures is given in parentheses.

tures of the two enzymes (Dijkstra et al., 1983) indicates that these sequence differences result in generally quite small differences in three-dimensional structure, in agreement with the limited NMR data as yet available. However, three local regions of structural difference were noted in the crystallographic study, of which the loop formed by residues 59–70 has assumed the greatest importance. There is only a single-residue difference in this region (Val → Phe-63), and yet the different conformations adopted by this loop in the two crystal structures are such that the position of Tyr-69 differs by 5 Å from one enzyme to the other. This conformational difference, observed in the crystal, has been implicated in the different micelle binding properties displayed by the bovine and porcine enzymes. However, such a structural difference between the two enzymes in solution is not apparent from the NMR studies reported here. Indeed, the observation of NOE's, of comparable intensities, between Tyr-69 and Tyr-52 in both bovine and porcine PLA_2 suggests that the solution-state conformations of the two enzymes are very similar in this region, despite the amino acid substitution in the loop 59–70. This difference between solution and crystal structures may cast doubt on the conclusions drawn about the functional role of this loop from the crystal data [see also Achari et al. (1987)].

A comparison of the chemical shifts of assigned resonances of the porcine enzyme and isoenzyme (Table I) shows that they are strikingly similar. This indicates that porcine iso-PLA_2, for which a crystal structure is not yet available, has a structure closely similar to that of porcine PLA_2.

Effects of Temperature, pH, and Calcium Concentration. Figures 6 and 7 illustrate the changes in the aromatic region of the ^{1}H NMR spectrum of bovine PLA_2 at pH* 7.1 produced by progressive addition of calcium ions, or by increasing the sample temperature in the absence of calcium. A number of

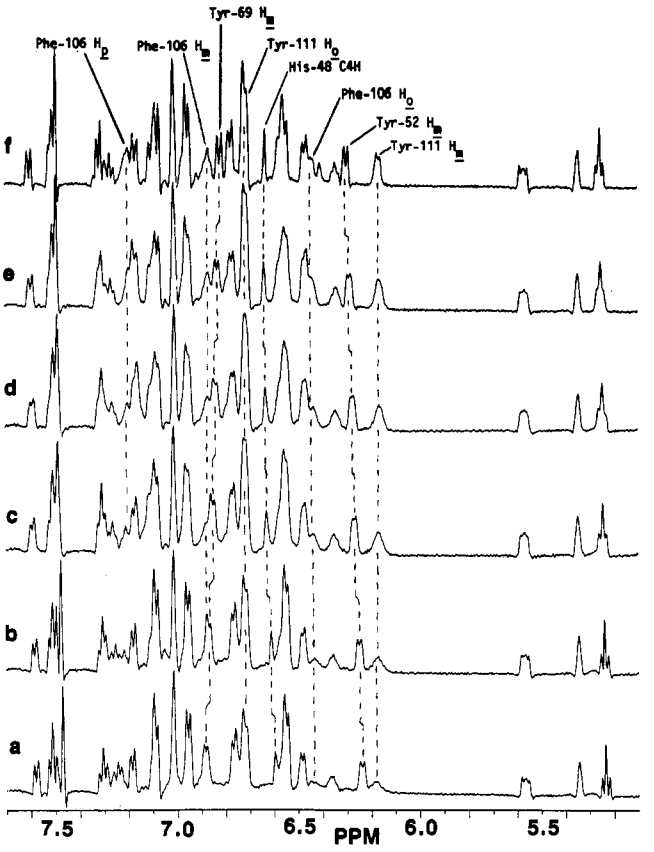

FIGURE 6: Aromatic region of the 500-MHz ^{1}H NMR spectrum of bovine PLA_2 (pH* 7.1, 320 K, constant ionic strength) at the following calcium ion concentrations: (a) 0, (b) 1, (c) 5, (d) 10, (e) 20, and (f) 50 mM.

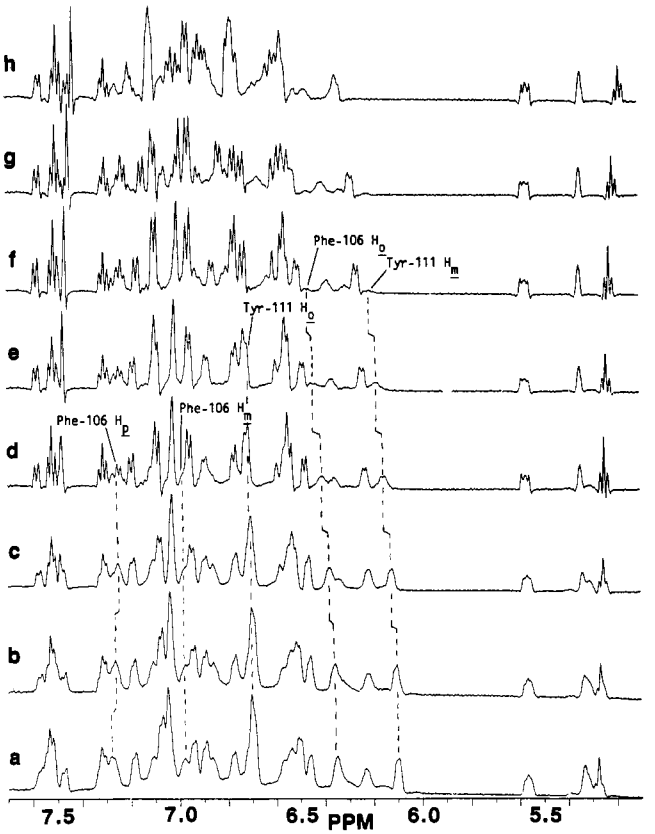

FIGURE 7: Aromatic region of the 500-MHz ^{1}H NMR spectrum of bovine PLA_2 (pH* 7.1, 300 mM NaCl, in the absence of calcium ions) at the following temperatures: (a) 300, (b) 305, (c) 310, (d) 315, (e) 320, (f) 325, (g) 330, and (h) 335 K.

resonances show significant changes in chemical shift as the calcium concentration is increased (Figure 6), notably those of the meta protons of Tyr-52 (6.29 ppm) and the meta protons of Tyr-69 (6.85 ppm). The C4H resonance of His-48 is also affected by the addition of calcium. Previous workers have shown that the pK_a of His-48 is lowered from 6.5 to 5.7 on addition of calcium ions (Aguiar et al., 1979). In this case, at pH* 7, we are observing a combination of the change in the pK_a of His-48 caused by the addition of Ca^{2+} and the direct effect of the calcium ion on the chemical shift of neutral His-48. A number of resonances show changes in chemical shift over the range 305–340 K; in particular, the resonances at the high-field edge of the aromatic region tend to shift downfield, toward their random coil positions.

In addition to these changes in chemical shift, the line width of certain residues is found to depend on both calcium concentration and temperature. (Deconvoluted spectra are presented in Figures 6 and 7 for clarity, but all line-width and intensity measurements have been made on spectra processed with no resolution enhancement.) The resonances of Tyr-111 and Phe-106 are broad at 320 K in the absence of calcium but sharpen markedly as the calcium concentration is increased, with only very slight changes in chemical shift. As indicated in Figure 6, this decrease in line width is discernible for both resonances of Tyr-111 and all three signals of Phe-106, and in the high-field region of the spectrum (not shown), the methyl resonances of Leu-41 show parallel behavior. An increase in temperature (Figure 7) is found to affect the line width of the same resonances. For example, the meta proton resonance of Tyr-111 at 6.10–6.15 ppm has a line width comparable to that of the other aromatic resonances at 305 K, but it is very broad at 320 K and almost undetectable at higher temperatures. As with calcium binding, the broadening of the aromatic signals of Phe-106 and Tyr-111 with increasing temperature is paralleled by that of the methyl resonances of Leu-41 (not shown) and the aromatic signal of Tyr-25 (resonance obscured at pH* 7, but its behavior can be followed at lower pH).

All four residues show similar effects of temperature in the presence of 50 mM $CaCl_2$, although the onset of the selective line broadening is shifted to somewhat higher temperatures than that in the absence of calcium. Line broadening is also observed as the pH is decreased in the absence of calcium, particularly below pH* 5.5 (not shown), although the effect is less noticeable since these resonances are fairly broad even at pH* 7.

The increase in line width with increasing temperature is a selective effect on the resonances of only four specific residues, Tyr-25, Leu-41, Phe-106 and Tyr-111, out of the 26 for which resonances have been assigned, and these residues are close together in the crystal structure of the protein. This behavior is characteristic of an exchange contribution to the line width, implying that the region of the protein in which these residues are found exists in two (or more) distinct conformational states. As we move to higher calcium concentrations or to lower temperatures the exchange regime moves from intermediate on the NMR time scale to slow. The marked decrease in line width produced by calcium binding could be explained by an effect on the relative populations of and/or on the rate of interconversion between the different conformational states. At the lower temperatures for which spectra are shown in Figure 7, the integral of the resonance of the meta protons of Tyr-111 is close to two protons, with or without calcium (see Table II). However, as the temperature is raised to around 320 K (particularly in the absence of calcium) the signal broadens, and the integral becomes significantly less than two protons, suggesting that there is an additional resonance elsewhere in the spectrum, corresponding to a second conformational state of this residue, with which the resonance at 6.1–6.2 ppm is in exchange. The population of this latter state is increasing as the temperature is increasing. We have not been able to observe this second, minor, resonance directly; its fractional population is very low at the temperatures where it would be expected to be sharp and does not appear to increase above 0.25 at higher temperatures, by which time it will have become very broad. An estimate of the rate of exchange between the two conformational states may however be made by subtracting the natural line width from the observed, exchange-broadened, line width. This gives estimates of the exchange rate of $\sim 9\ s^{-1}$ at pH* 7.1, 310 K, and $\sim 25\ s^{-1}$ at pH* 7.1, 315 K.

Table II: Intensity and Line Width of the C_mH Resonance of Tyr-111 of Bovine PLA_2 as a Function of pH, Temperature, and Calcium Concentration[a]

pH*	temp (K)	$[Ca^{2+}]$ (mM)	line width (Hz)	intensity (protons)
5.2	305	50	18	1.8
5.2	310	50	20	1.7
5.2	315	50	22	1.4
5.2	320	50	27	1.3
7.1	310	0	21	1.7
7.1	310	50	18	1.9
7.1	315	0	26	1.3
7.1	315	50	17	1.7

[a] Resonance intensity was measured relative to the Cys-77 $C_\alpha H$ resonance at 5.58 ppm (taken as one proton) and to the most downfield part of the aromatic region comprising resonances from Trp-3 C_2H, C_4H, and C_7H and Phe-94 C_mH (taken as five protons). Line width was measured at half-height.

The resonances of Leu-41, Phe-106, and Tyr-111 can also be clearly assigned in the spectrum of the porcine enzyme (see Table I). They have closely similar chemical shifts to those seen for the bovine enzyme, with the sole exception of the ortho proton signal of Phe-106, and also, importantly, very similar patterns of NOE connectivities. This indicates, in agreement with the crystallographic results (Dijkstra et al., 1983), that this part of the structure is very similar in the protein from the two sources. However, the effects of temperature and calcium binding on the line widths of these resonances are much less marked in the spectra of the porcine enzyme. For example, the resonances of Tyr-111 in porcine PLA_2 are always sharp at 320 K, in the presence or absence of calcium ions, and they stay sharp over the pH* range 3–7. Thus, the rate and/or the equilibrium constant for the conformational equilibrium detected in the bovine enzyme differs in the porcine enzyme in such a way as to make its effects in the NMR spectrum much smaller.

In the spectra of porcine iso-PLA_2, which differs from porcine PLA_2 by just four amino acids, the resonances of Tyr-111 behave more like the corresponding signals of bovine PLA_2 than those of porcine PLA_2. As in bovine PLA_2, the resonances of Tyr-111 are found to sharpen and move slightly upfield on calcium binding. The temperature dependence of the line broadening in the absence and presence of calcium ions is more difficult to quantify as a result of partial resonance overlap. However, it is clear that there are changes in line width of the resonances of Leu-41, Phe-106, and Tyr-111 in porcine iso-PLA_2 which are not apparent in porcine PLA_2 and that these are affected in the same way by the same changes in experimental conditions as described above for the bovine enzyme.

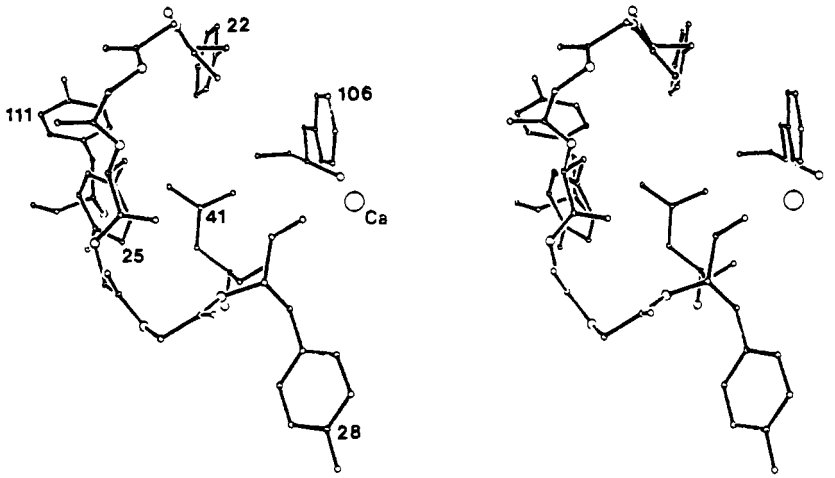

FIGURE 8: Stereoview of part of the crystal structure of bovine PLA_2 [from the coordinates of Dijkstra et al. (1981)] showing the relative orientations of the side chains of residues Tyr-25, Tyr-28, Tyr-111, Phe-22, Phe-106, and Leu-41 as well as the position of the essential calcium ion.

These observations provide clear evidence for the existence of a local conformational equilibrium in bovine phospholipase A_2 in solution. As yet, we can only identify the part of the structure involved as that containing Tyr-25, Leu-41, Phe-106, and Tyr-111 (Figure 8); we cannot specify the precise nature of the equilibrium. Some preliminary indications do, however, come from the comparisons with porcine PLA_2 and iso-porcine PLA_2. Few of the 20 amino acid differences between porcine and bovine PLA_2 are close to the relevant part of the structure; one of these is at position 17, which is on a short stretch of α-helix, lying between the residues of interest and the solvent. In porcine PLA_2, residue 17 is a histidine; in bovine PLA_2, it is a glutamate. One of only four sequence differences between porcine PLA_2 and iso-PLA_2 also occurs at position 17, which is an aspartate in the isoenzyme. Thus, there appears to be a correlation between the presence of an acidic residue at position 17 and the manifestation of a conformational equilibrium in the NMR spectrum; this suggests that the helix containing residue 17 may be involved in the equilibrium.

Recently, de Haas et al. (1987) studied this region of the enzyme in connection with the binding properties observed for the phospholipases A_2. The "lipid binding domain", a predominantly hydrophobic region around the active site, contains three cationic residues in porcine PLA_2: Arg-6, Lys-10, and His-17. Anionic phospholipid substrates and substrate analogues bind to this domain well below their critical micelle concentration, leading to protein–lipid aggregation into high molecular weight complexes. As noted above, residue 17 is anionic in both bovine PLA_2 and porcine iso-PLA_2. [The crystal structure of the bovine enzyme (Dijkstra et al., 1981) indicates the possibility of a salt bridge between Lys-10 and Glu-17, although since these are surface residues, this would probably be weak in solution.] From comparisons of the behavior of the porcine enzyme and isoenzyme, de Haas et al. (1987) concluded that the state of protonation of the amino acid at position 17 strongly influences the tendency of the enzyme to form high molecular weight complexes in the presence of substrate analogues. Indeed, they concluded that the trend in the formation of the high molecular weight protein–lipid complexes (porcine > isoporcine > bovine) correlates with the total positive charge of the lipid binding domain. The evidence presented in this paper shows that this correlation extends to a conformational equilibrium in a region of the protein close to residue 17; it remains to be established whether a causal relationship underlies this correlation with micelle binding.

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SUPPLEMENTARY MATERIAL AVAILABLE

Assignment of aromatic and associated resonances of bovine PLA_2 and one figure depicting the aromatic region of the 500-MHz 2-D COSY ^{1}H NMR spectrum of amidinated bovine PLA_2 and Phe^5-$^2H_5$-labeled amidinated bovine PLA_2 (14 pages). Ordering information is given on any current masthead page.

Registry No. PLA_2, 9001-84-7.

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^{1}H NMR Studies of Porcine Calbindin D_{9k} in Solution: Sequential Resonance Assignment, Secondary Structure, and Global Fold†

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ABSTRACT: The ^{1}H nuclear magnetic resonance (NMR) spectrum of Ca^{2+}-saturated porcine calbindin D_{9k} (78 amino acids, M_r 8800) has been assigned. Greater than 98% of the ^{1}H resonances, including spin systems for each amino acid residue, have been identified by using an approach that integrates data from a wide range of two-dimensional scalar correlated NMR experiments [Chazin, Rance, & Wright (1988) *J. Mol. Biol. 202*, 603-626]. Due to the limited quantity of sample and conformational heterogeneity of the protein, two-dimensional nuclear Overhauser effect (NOE) experiments also played an essential role in the identification of spin systems. On the basis of the pattern of scalar connectivities, 43 of the 78 spin systems could be directly assigned to the appropriate residue type. This provided an ample basis for obtaining the sequence-specific resonance assignments. The elements of secondary structure are identified from sequential and medium-range NOEs, values of $^3J_{NH\alpha}$, and the location of slowly exchanging backbone amide protons. Four well-defined helices and a mini β-sheet between the two calcium binding loops are present in solution. These elements of secondary structure and a few key long-range NOEs provided sufficient information to define the global fold of the protein in solution. Generally good agreement is found between the crystal structure of the minor A form of bovine calbindin D_{9k} and the solution structure of intact porcine calbindin D_{9k}. The only significant difference is a short one-turn helix in the loop between helices II and III in the bovine crystal structure, which is clearly absent in the porcine solution structure.

Calbindin D_{9k} (formerly intestinal calcium binding protein) is a small, acidic, and very heat stable protein found in the small intestine of all mammalian species so far examined (Kallfelz et al., 1967; Drescher & De Luca, 1971; Hitchman & Harrison, 1972; Taylor, 1983). It belongs to the troponin C superfamily of calcium regulatory proteins that are characterized by a common helix-loop-helix motif for the calcium binding sites, termed EF-hands (Kretsinger, 1972). These proteins exhibit changes in conformation in response to binding of metal ions, the presumed structural basis for function. While the synthesis of calbindin D_{9k} has been shown to be vitamin D dependent (Corradino et al., 1976), its function is not well understood. It is agreed that the protein is involved in Ca^{2+} ion transport (Wasserman et al., 1983) or as a Ca^{2+} reservoir or buffer (Mayel-Afsharet, 1988). The X-ray crystal structure of the minor A form of the bovine calbindin D_{9k} has been refined to 2.3-Å resolution (Szebenyi & Moffat, 1986). Recently, the gene encoding for the bovine protein has been synthesized and expressed in *Escherichia coli* (Brodin et al., 1986), allowing for study by protein engineering and biophysical methods (Linse et al., 1987, 1988; Wendt et al., 1988; Forsén et al., 1988).

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