Side-Chain Dynamics of Two Aromatic Amino Acids in Pancreatic Phospholipase A_2 As Studied by Deuterium Nuclear Magnetic Resonance†

P. R. Allegrini,‡ G. J. M. van Scharrenburg,§ A. J. Slotboom,§ G. H. de Haas,§ and J. Seelig*,‡

Department of Biophysical Chemistry, Biocenter, University of Basel, Basel, Switzerland, and Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, "De Uithof", Utrecht, The Netherlands

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ABSTRACT: The flexibility of individual amino acid side chains of pancreatic phospholipase A_2 in aqueous and micellar solutions was studied with deuterium nuclear magnetic resonance (^2H NMR). Bovine pancreatic phospholipase A_2 was selectively deuterated at the aromatic ring systems of Trp-3 and Phe-5 and porcine pancreatic phospholipase A_2 at Trp-3 only. Solid-state ^2H NMR spectra of the lyophilized enzymes exhibited quadrupole splittings on the order of 130 kHz, indicating almost complete immobilization of the aromatic ring systems. Exposure to a water-saturated atmosphere did not remove these steric constraints. However, side-chain mobility could be induced for the tryptophyl residue of the bovine enzyme by dissolving this enzyme in aqueous buffer or micellar solution whereas the phenyl ring always remained immobile and served as a probe for the protein's overall rotation. Typical correlation times for the tryptophyl and phenyl aromatic ring systems in aqueous solution were 7 ps and 13 ns (at 20 °C), respectively. The correlation time of the phenyl ring was longer than expected for the monomeric protein (~6 ns), suggesting some aggregation of the protein at the high concentrations used for the NMR measurements. Addition of a micellar solution of oleoylphosphocholine had no influence on the motional freedom of the tryptophyl residue but approximately doubled the correlation time of the phenyl ring, indicating an increase of the effective volume of the tumbling particle due to lipid–protein interaction. A different behavior was observed for the Trp-3 residue of porcine phospholipase A_2. In aqueous solution, this residue was characterized by a broad resonance with a correlation time of about 13 ns (at 20 °C) which indicated immobilization of this residue in the protein structure and also partial aggregation of the enzyme. Upon addition of oleoylphosphocholine micelles, the ^2H NMR spectrum completely disappeared. This is explained by the formation of a large protein–lipid complex which broadens the ^2H NMR line width beyond detectability.

The biological membrane is a mixture of lipids and proteins, and knowledge of the dynamic properties of both components is of considerable interest in developing models for membrane function. Nuclear magnetic resonance (NMR)[1] provides a most general experimental technique to observe a broad range of molecular motions, and ^2H NMR in particular has led to a distinct improvement in our knowledge of lipid dynamics in bilayer membranes [for reviews, cf. Seelig & Seelig (1980) and Davis (1983)]. Using selectively deuterated lipids, ^2H NMR has yielded a detailed description of the lipid conformation and the rates of hydrocarbon chain and polar headgroup motions. By employing membrane reconstitution procedures, it has also been possible to monitor the influence of integral membrane proteins on the lipid conformation (Seelig et al., 1981, 1982; Tamm & Seelig, 1983; Rice et al., 1979; Paddy et al., 1981).

In the present study, we have addressed the complementary question of how the internal flexibility of a protein can be influenced by interaction with an organized lipid–water interface. This problem was approached by selectively deuterating phospholipase A_2 and monitoring the dynamic behavior of the amino acid side chains in the presence and absence of lipids.

The catalytic activity of phospholipase A_2 consists in cleaving off the *sn*-2 fatty acyl chain of phospholipids. Characteristic features of extracellular phospholipases from pancreatic juice and snake or insect venom are their low molecular weight (~14000), a requirement for Ca^{2+} ions for activity, and a high stability under denaturing conditions. The latter may be explained by the presence of seven disulfide bridges [for a review, cf. Slotboom et al. (1982)]. Furthermore, and most important for NMR studies, pancreatic enzymes can be purified in large quantities.

The complete amino acid sequences of both the porcine (Puijk et al., 1977) and bovine (Fleer et al., 1978) enzymes are known. The two enzymes show a high homology in primary structure. They are secreted as almost inactive proenzymes which are activated by limited trypsinolysis. The activation of the phospholipases is accompanied by a conformational change in the N-terminal region of the protein.

An important step toward a detailed description of the mechanism of action of pancreatic phospholipase A_2 has been determination of the three-dimensional structure of the bovine enzyme by X-ray diffraction analysis with a resolution of 1.7 Å (Dijkstra et al., 1981a; Verheij et al., 1980). The molecule has the shape of a flattened cylinder with dimensions of approximately 22 × 30 × 42 Å^3. About 50% of the amino acids are in the α-helix conformation and about 10% in the β-structure. Recently, the X-ray structure of the porcine enzyme has also been solved (Dijkstra et al., 1983).

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‡ University of Basel.

§ State University of Utrecht.

[1] Abbreviations: IRS, interface recognition site; NMR, nuclear magnetic resonance; AMPA, fully ϵ-amidinated phospholipase A_2.

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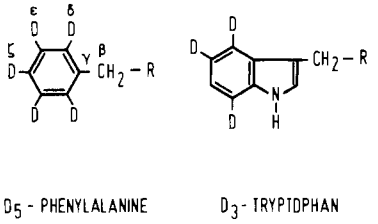

FIGURE 1: Deuteration sites of amino acid side chains Trp-3 and Phe-5.

The activity of pancreatic phospholipase A_2 is highly dependent on the physical state of the substrate. The observation that the hydrolysis rate of the enzyme is distinctly increased if the substrate is present in an aggregated form has led to the hypothesis of a specific interface recognition site (IRS), separate from the active site (Verger et al., 1973; Pieterson et al., 1974; Brockerhoff, 1973). On the basis of the X-ray structural analysis, Dijkstra et al. (1981b) described the interfacial recognition site of bovine phospholipase A_2 in terms of three subdomains containing mainly hydrophobic amino acids of the N-terminal region, which is consistent with earlier spectroscopic evidence (van Dam-Mieras et al., 1975). One of these amino acids residues, namely, Trp-3, was therefore specifically deuterated in this study.

The other amino acid residue investigated, Phe-5, is located in the same α-helical structure but directed toward the active site in the interior of the protein. The functional role of Phe-5 is not clear yet, but since it is part of the hydrophobic wall surrounding the active center, it may well be involved in the binding of the substrate monomer. Substitution of Phe-5 by Tyr in bovine phospholipase A_2 destroys the active site but does not affect the lipid binding properties (van Scharrenburg et al., 1982).

Using ^2H NMR, we have studied the protein mobility and side-chain flexibility of deuterated bovine and porcine phospholipases in the lyophilized state, in aqueous solution, and in the presence of micellar substrates.

Materials and Methods

Phenylalanine which was perdeuterated at the aromatic ring system (Figure 1) was purchased from Merck Sharp & Dohme, Canada. Bovine and porcine pancreatic prophospholipases A_2 were purified from pancreatic tissue and converted into the corresponding phospholipases A_2 by limited proteolysis as described by Fleer et al. (1978) and Nieuwenhuizen et al. (1974), respectively. Bovine phospholipase A_2 concentrations were calculated from the absorbance at 280 nm with an $E^{1\%}_{1cm}$ value of 12.5. Enzymatic activities were routinely determined by the titrimetric assay procedure using egg yolk lipoproteins as substrates (Nieuwenhuizen et al., 1974). In contrast to the described procedure, a 2-fold higher Ca^{2+} concentration and a 1.5-fold higher sodium deoxycholate concentration were used. *cis*-9-Octadeceylphosphocholine was synthesized as described by van Dam-Mieras et al. (1975).

Bovine Phe^5-$^2H_5$-Labeled ε-Amidinated Phospholipase A_2. A phenylalanine deuterated at all phenyl ring positions was incorporated at position 5 of bovine ε-amidinated phospholipase A_2 (AMPA) by a semisynthetic pathway (van Scharrenburg et al., 1981). The deuterated bovine AMPA was indistinguishable from native AMPA with respect to catalytic, substrate binding, and Ca^{2+} binding properties. Bovine ε-amidinated phospholipase A_2 possesses approximately 75% of the enzymatic activity of the native phospholipase A_2 on micellar dioctanoyllecithin, and the Ca^{2+} and substrate binding properties of both enzymes are identical.

Bovine and Porcine [Trp^3-$^2H_3$]Phospholipases A_2. The lyophilized phospholipases A_2 were treated for 2 h under nitrogen at room temperature with CF_3COOD (Bak et al., 1969; Holt et al., 1971). Subsequently, the enzymes were exhaustively dialyzed against 0.1% acetic acid in order to remove all possibly present deuterium-labeled small molecules. After lyophilization, the proteins were dissolved in a 5 mM acetate buffer, pH 6.0, and subjected for 5 min to a temperature of 80 °C in order to back-exchange the amide protons (Campbell et al., 1975). After this heat treatment, the phospholipases A_2 were purified on CM-cellulose as described before (Fleer et al., 1978; Nieuwenhuizen et al., 1974). The fractions containing the deuterated phospholipases A_2 were pooled, again exhaustively dialyzed against distilled H_2O, and lyophilized. The purified enzymes possessed full enzymatic activities. A ^1H NMR (360-MHz) spectrum of bovine [Trp^3-$^2H_3$]-phospholipase A_2 was obtained with a Bruker HX-360 spectrometer at the S.O.N.-Facility in Groningen. The aromatic region of the spectrum showed a decrease of approximately 70% of the intensities of the H-4, H-5, and H-7 proton resonances of the Trp-3 residue, proving the labeling of this unique Trp residue in phospholipase A_2. The above described procedure has been shown to be specific for the labeling of exclusively Trp residues in various proteins (Bak et al., 1969; Holt et al., 1971). To unambiguously exclude the presence of any ^2H-labeled amino acid residues other than the unique Trp-3 in phospholipase A_2, we applied the same labeling procedure to des(Ala^1–Met^8) bovine phospholipase A_2 (van Scharrenburg et al., 1981). This C-terminal protein fragment consists of 115 amino acid residues and lacks the N-terminal octapeptide H_2N-Ala-Leu-Trp-Gln-Phe-Asn-Gly-Met. As had to be expected, the Trp lacking des(Ala^1–Met^8)phospholipase A_2 did not express any detectable ^2H NMR signal. Therefore, these findings convincingly demonstrate that in the phospholipases A_2 exclusively Trp^3 has been deuterated and that no deuterated small molecules were present.

^2H NMR Measurements. NMR samples were prepared as follows. About 20 mg of deuterium-labeled phospholipase A_2 was dissolved in 0.5 mL of deuterium-depleted buffer (50 mM sodium acetate/acetic acid, pH 6.0, containing 100 mM NaCl) and filled either in a thick-walled test tube (8-mm o.d.) for T_1 measurements using a solenoid probe head or in a conventional NMR sample tube for recording high-resolution ^2H NMR spectra with a high-resolution probe head (Helmholtz coils). To study the lipid–protein interaction, 20 mg of oleoylphosphocholine and 7.4 mg of $CaCl_2 \cdot 2H_2O$ yielding a final $CaCl_2$ concentration of 0.1 M were added to the phospholipase sample. The critical micellar concentration of oleoylphosphocholine is 7 μM (Donné-Op den Kelder et al., 1981).

All ^2H NMR measurements were made on a Bruker-Spectrospin CXP-300 spectrometer operating at 46.1-MHz deuterium frequency. The experimental conditions are similar to those described earlier (Tamm & Seelig, 1983). Solid-state spectra were recorded with a solenoid coil using the quadrupole echo technique (Davis et al., 1976); 90° pulses of 4–5-μs duration were used with an echo separation of 30 μs. T_1 measurements of aqueous solutions were made with the same probe head. The recovery of the magnetization after a 180° inverting pulse was followed by using the quadrupole echo. Since the solenoid probe head could not be shimmed better than about 100 Hz, all line-width measurements were made with a high-resolution probe head (no lock signal); 90° pulses of 25-μs length were used in this case.

Results

^2H NMR Studies on Solid Phospholipase A_2. Using the quadrupole echo technique (Davis et al., 1976) ^2H NMR

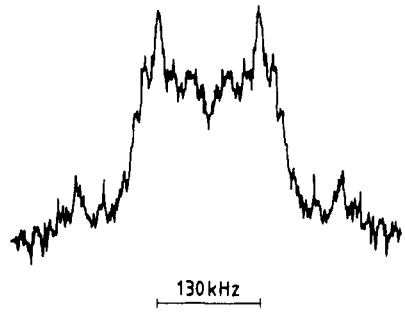

FIGURE 2: Solid-state ^2H NMR spectrum of Trp-3-deuterated bovine pancreatic phospholipase A_2. Conditions: 85 mg of lyophilized enzyme measured at 25 °C; spectral width 500 kHz; recycle delay 5 s; 37 000 scans. The spectrum was measured with quadrature detection and symmetrized to improve the signal to noise ratio.

spectra can be recorded of peptides and proteins in the solid or semisolid state. The line shape of the solid-state ^2H NMR spectrum is particularly sensitive to ring motions of aromatic amino acid side chains (Rice et al., 1981; Gall et al., 1981; Kinsey et al., 1981). Three extreme cases of ring motion can be discerned: (i) no ring motion at all, (ii) 2-fold jumps whereby the aromatic rings execute 180° reorientational flips, and (iii) rapid rotational ring diffusion. We were interested in which types of motions occur in lyophilized phospholipase A_2.

Figure 2 shows the ^2H NMR spectrum of lyophilized bovine pancreatic phospholipase A_2, selectively deuterated at Trp-3. Since only 85 mg (6 μmol) of enzyme was available, the rather low signal to noise ratio of the powder pattern prohibits a detailed line-shape simulation. However, the separation of the most intense peaks in the spectrum can easily be measured and amounts to approximately 130 kHz. On the basis of a static quadrupole coupling constant of 183 kHz for the aromatic deuterons of tryptophan (Rice et al., 1981), a maximum separation of 137 kHz can be calculated for a completely rigid Trp-3 side chain. Hence, Figure 2 demonstrates an almost complete immobilization of the tryptophyl residue. ^2H NMR spectra were also recorded for the Phe-5-labeled bovine enzyme and the Trp-3-labeled porcine enzyme. Both phospholipases yielded powder patterns with about 130-kHz separation, indicating that both ring systems were also immobilized in the lyophilized protein. This situation was not changed when both Trp-3-labeled enzymes were exposed to a water-saturated atmosphere for 24 h.

Aqueous Solutions of Phospholipase A_2. The pancreatic phospholipases are water-soluble enzymes. For a globular protein with a molecular weight of 14 000, the rotational diffusion constant can be predicted to be about $D_r = 10^7$–10^8 s^{-1}, yielding a characteristic correlation time $\tau_c = (6D_r)^{-1} =$ 20–2 ns. Due to this rapid isotropic tumbling, the quadrupole splitting will be averaged out, and the ^2H NMR spectrum will consist of a single resonance. However, dynamic information can still be extracted from measurments of the spin–lattice (T_1) relaxation time and the line width as has been demonstrated recently for lysozyme (Wooten & Cohen, 1979; Schramm & Oldfield, 1983). The main advantage of using ^2H NMR in these experiments stems from the fact that the dominant relaxation pathway is quadrupolar relaxation, and the molecular interpretation of the T_1 relaxation time and the line width is thus governed by rather simple formalisms.

The outcome of two typical T_1 relaxation time measurements with bovine and porcine phospholipase A_2 labeled at the same amino acid, Trp-3, is displayed in Figure 3. The bovine enzyme is characterized by a sharp line with a long T_1 relaxation time (T_1 = 300 ms at 20 °C) whereas the porcine

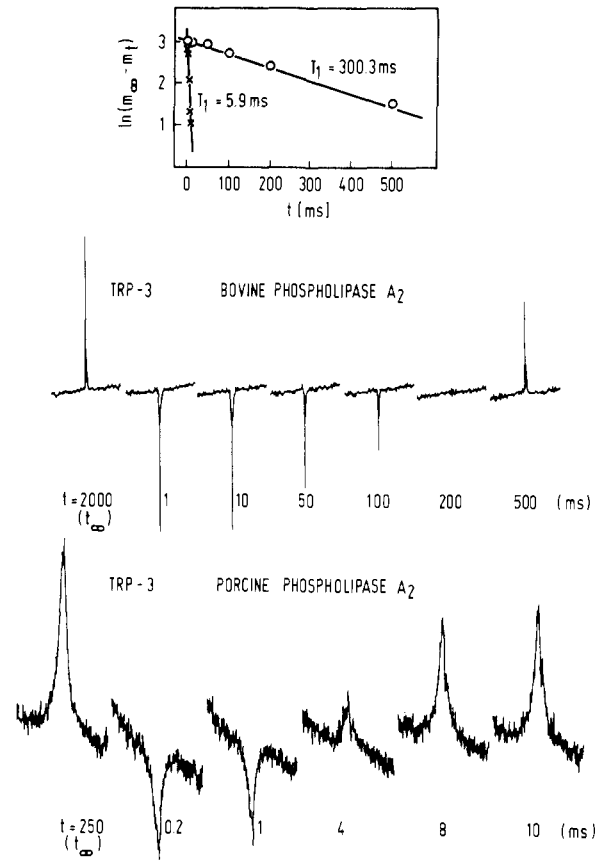

FIGURE 3: Measurement of deuterium spin–lattice (T_1) relaxation time of Trp-3-labeled bovine (○) and porcine (×) phospholipase A_2 in aqueous solution. Approximately 20 mg of phospholipase was dissolved in 0.5 mL of deuterium-depleted buffer. Conditions: spectral width 10 kHz; 1000 scans per spectrum. Spectra were measured at 20 and 25 °C for the bovine and porcine enzymes, respectively.

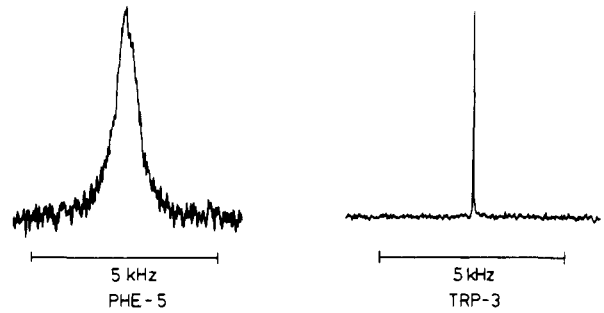

FIGURE 4: High-resolution ^2H NMR spectra of aqueous solutions of bovine phospholipase A_2 deuterated at Trp-3 and Phe-5, respectively. Measuring temperature, 25 °C. Forty milligrams of the protein was dissolved in 1 mL of deuterium-depleted buffer; 2500 and 10 000 scans were recorded for the Trp-3- and Phe-5-deuterated proteins, respectively.

enzyme gives rise to a broad line with a short T_1 (T_1 = 5.9 ms at 25 °C).

A comparison of two different amino acids, Trp-3 and Phe-5, within the *same* phospholipase A_2 (bovine) is reproduced in Figure 4. Again one notes a considerable difference in line width between the two aromatic residues, indicating distinct motional differences. Spectral simulations were carried out for the broad resonances (assuming Lorentzian line shapes) in order to obtain a more precise estimate of the line width. Table I provides a summary of the line widths and T_1 relaxation time data of the various amino acid residues investigated.

The interaction of phospholipase A_2 with a lipid substrate was investigated by adding oleoylphosphocholine and $CaCl_2$

in solid form to the aqueous solution of the enzyme. The resulting solution was characterized by the following concentrations: phospholipase A_2, 2.9 mM; oleoylphosphocholine, 89.5 mM; and $CaCl_2$, 100 mM. In the presence of Ca^{2+}, the enzyme binds to the micellar surface as has been demonstrated previously for various *n*-alkylphosphocholine analogues (de Araujo et al., 1979; Hille et al., 1981; Donné-Op den Kelder, 1981). Table I shows a comparison of the T_1 relaxation times and the line widths with and without micelles as a function of temperature. No general conclusions can be drawn from Table I since the addition of micelles produces different effects for the three residues investigated. Trp-3 bovine phospholipase A_2 exhibits practically no difference between aqueous and micellar solution, while the Phe-5 residue of the same enzyme experiences an almost 2-fold increase in the T_1 relaxation time and also an increase in line width. Finally, the Trp-3 resonance of the porcine enzyme is broadened beyond detection in the presence of micelles. The latter process is reversible since the ^2H NMR signal can be recovered by removing the micelles with gel exclusion chromatography (Slotboom et al., 1977).

Discussion

^2H NMR is well suited for studying the mobility in a protein. A prerequisite for obtaining meaningful results is the synthesis of a selectively deuterated protein which was achieved here for two different aromatic amino acid side chains.

The measurements with the lyophilized phospholipase A_2 demonstrate that 6 μmol of enzyme is sufficient to yield ^2H NMR solid-state spectra of reasonable quality. All three enzymes investigated exhibit powder patterns with a 130-kHz splitting, indicating an almost complete immobilization of the aromatic ring systems. Furthermore, it was not possible to induce the rotation of the Trp-3 residue by exposing the phospholipase A_2 to a water-saturated atmosphere even though the same residue is freely rotating in the dissolved enzyme, at least for the bovine enzyme.

If one assumes isotropic reorientation characterized by a single correlation time τ_c, then, as is well established (Abragam, 1961)

$$\frac{1}{T_1} = \frac{3}{20}\pi^2\left[\frac{e^2qQ}{h}\right]^2\left[1+\frac{\eta^2}{3}\right]\left[\frac{2\tau_c}{1+\omega_0^2\tau_c^2}+\frac{8\tau_c}{1+4\omega_0^2\tau_c^2}\right] \quad (1)$$

and

$$\Delta\nu_{1/2} = \frac{1}{\pi T_2} = \frac{3}{20}\pi\left[\frac{e^2qQ}{h}\right]^2\left[1+\frac{\eta^2}{3}\right]\times\left[3\tau_c+\frac{5\tau_c}{1+\omega_0^2\tau_c^2}+\frac{2\tau_c}{1+4\omega_0^2\tau_c^2}\right] \quad (2)$$

where η is the asymmetry parameter, ω_0 the Larmor precessional frequency, and $\Delta\nu_{1/2}$ the line width at half-height. The spin–lattice relaxation curve exhibits a minimum around $\tau_c \sim \omega_0^{-1}$, and a single T_1 measurement is usually not sufficient to allow an unambiguous determination of τ_c. However, in combination with the line width or the measurement of the temperature dependence of T_1, the assignment of a unique τ_c value becomes straightforward.

Bovine Phospholipase A_2. Inspection of Table I reveals that Phe-5-deuterated bovine phospholipase A_2 exhibits a negative temperature dependence in T_1; i.e., increasing temperatures lead to shorter T_1 relaxation times. This behavior is typical for the so-called slow correlation time regime where $\omega_0 > \tau_c^{-1}$, and eq 1 predicts correlation times (τ_c) in the range of 12–25 ns, assuming a quadrupole coupling constant of 180 kHz and neglecting the asymmetry parameter ($\eta \simeq 0.03$; Rice et al., 1981). Insertion of the calculated τ_c values into the line-width eq 2 yields a very good agreement with the experimentally observed line widths listed in Table I.

The correlation time τ_c is related to the effective volume V_{eff} of the reorienting sphere according to

$$\tau_c = \frac{V_{eff}\eta_0}{kT} \quad (3)$$

where η_0 is the viscosity of the solution, k the Boltzmann constant, and T the absolute temperature. The viscosity of the phospholipase A_2 solution was not measured directly but can be estimated by comparison with solutions of ribonuclease A which has a similar molecular weight [cf. Buzzell & Tanford (1956)]. For a phospholipase concentration of 40 mg/mL, which is typical for the NMR experiments, we estimate a viscosity of $\eta_0 = 1.3$ cP (at 20 °C), which together with $\tau_c = 13.5$ ns yields an effective volume of 4.2×10^{-20} cm^3. On the basis of the crystallographic dimensions, the total volume of the protein is predicted to be 2.8×10^{-20} cm^3. As a first conclusion, it therefore follows that the phenyl ring is embedded rigidly in the protein and acts as a probe for the overall protein rotation.

The effective volume as determined by NMR is larger than that calculated from the crystallographic data. This difference may be explained by the hydration of the enzyme in solution, by the uncertainty in the estimated viscosity, and perhaps by a partial self-association of phospholipase A_2. It is noteworthy in this respect that lysozyme, an enzyme of similar molecular weight (13 900), exhibits correlation times from 6 ns (monomeric state) to about 23 ns depending on the state of aggregation of the enzyme [Wooten & Cohen, 1979; cf. also p 479 of Jardetzky & Roberts (1981)].

A completely different behavior is observed for the Trp-3 residue of bovine phospholipase. In solution, this residue gives rise to a rather narrow line of less than 5 Hz line width which together with the positive temperature dependence of T_1 indicates a motional behavior typical for the fast correlation time regime ($\omega_0 << \tau_c^{-1}$). The correlation time τ_c is approximately 10 ps which is 3 orders of magnitude shorter than the correlation time of the Phe-5 ring. Similar short correlation times are obtained for small molecules in aqueous solution [cf. Mantsch et al. (1977) and Jardetzky & Roberts (1981)]. Hence, as a second result, it follows that the Trp-3 ring is moving freely in solution and is essentially unaffected by the attachment of the amino acid to the phospholipase polypeptide backbone.

We may now discuss the properties of bovine phospholipase A_2 in micellar solutions of oleoylphosphocholine. As indicated in Table I, the correlation time τ_c of the Phe-5 ring increases by almost a factor of 2 compared to that of the aqueous solution. This means not only that the phenyl ring remains immobilized but also that the effective volume of the tumbling molecule is considerably increased due to the binding of lipid molecules to the protein. The doubling of the effective volume can be explained quantitatively by assuming that each protein binds 30–50 molecules of oleoylphosphocholine (M_r 447).

This conclusion is consistent with earlier physicochemical studies on porcine pancreatic phospholipase A_2. According to Donné-Op den Kelder et al. (1981), the porcine phospholipase A_2 forms complexes with saturated *n*-alkylphosphocholines which are generally composed of 2 protein molecules and 60–100 lipid monomers [cf. also de Araujo et al. (1979)

Table I: Spin–Lattice Relaxation Time (T_1), Line Width at Half-Height ($\Delta\nu_{1/2}$), and Correlation Time (τ_c) of Phospholipase A_2 Solutions

		temp (°C)	T_1 (ms)[a]	$\Delta\nu_{1/2}$ (Hz)[b]	τ_c (ns)[c]	E_a (kJ/mol)[d]
Phe-5 bovine phospholipase A_2	aqueous solution	0	10.3	1200	23.3	
		10	7.2	790	16.1	14.0
		20	6.1	670	13.5	
		25		630	12	
	micellar solution	10	14.4		32.7	
		20	13.7		31.2	
		30	11.3		25.6	7.1
		40		1200	25.3	
		50		1100	23.0	
Trp-3 bovine phospholipase A_2	aqueous solution	0	170		0.0119	
		10	203	<5	0.0099	21.7
		20	290		0.0070	
		30	432		0.0047	
	micellar solution	0	164		0.0123	
		10	263	<5	0.0076	20.8
		20	304		0.0066	
Trp-3 porcine phospholipase A_2	aqueous solution	5	6.7		15.4	
		15	5.6		12.7	
		25	5.9	650	13.4	
	micellar solution[e]					

[a] T_1 measurements were made with a solenoid probe head and 4.5-μs (90°) pulses. The intrinsic field inhomogeneity was about 100–200 Hz. [b] Line width at half-height. Line-width measurements were made with a high-resolution probe head, but without a lock signal. As tested with ^2H_2O samples, the line width was less than 2 Hz. [c] Correlation times were evaluated from eq 1 and 2. Quadrupole coupling constants of 180 and 183 kHz were assumed for the aromatic deuterons of the phenylalanine and the tryptophan residue, respectively. The asymmetry parameter was neglected since $\eta < 0.05$ and enters only as η^2 in the relaxation formulas. [d] Activation energies (E_a) were determined from an Arrhenius plot of the correlation times (τ_c). [e] Resonance too broad; no signal observable.

and Hille et al. (1981)]. As the only exception, oleoylphosphocholine was found to form lipid–protein aggregates consisting of 3 porcine phospholipase A_2 molecules and 100 lipid monomers. However, bovine phospholipase A_2 may have different lipid binding properties than the porcine enzyme, and a complex of 2 bovine phospholipases A_2 and 60–100 lipid monomers of oleoylphosphocholine is in better agreement with the ^2H NMR results.

The correlation times determined by ^2H NMR furthermore indicate that the individual monomers are not interacting strongly but appear to rotate independently with their respective lipid shells. The correlation times of the whole complex can be estimated to be at least a factor of 2 larger than the measured correlation times.

The T_1 relaxation time of the Trp-3 residue is not altered upon binding of phospholipase A_2 to micelles. The correlation time amounts to about 12–7 ps with and without micelles which suggests that the tryptophan aromatic ring system of the bovine enzyme remains in the water phase and rotates freely without any additional restrictions.

Porcine Phospholipase A_2. Only the Trp-3, but not the Phe-5, ring was deuterated in this enzyme. However, the behavior of Trp-3 of porcine phospholipase A_2 is clearly different from that of the bovine enzyme (cf. Table I). In aqueous solution, the Trp-3 ring gives rise to broad resonances with $\Delta\nu_{1/2} \simeq 650$ Hz. From this and from the measurement of the spin–lattice relaxation T_1, a correlation time $\tau_c = 12$–15 ns can be derived which is again characteristic of the reorientation of the protein as a whole. Thus, in a 3 mM solution of the porcine enzyme, the Trp-3 ring is immobilized in the protein structure which may be contrasted with the free rotation of Trp-3 in the bovine enzyme under the same conditions. Moreover, upon addition of micelles, the Trp-3 resonance is no longer observable which suggests the formation of an even larger lipid–protein complex with a correlation time of $\tau_c > 40$ ns.

In conclusion, the ^2H NMR studies thus have demonstrated that (1) there can be dramatic differences between the mobility of amino acid side chains, viz., Trp-3 and Phe-5, which are located on the same stretch of α-helix, (2) the same residue (Trp-3) can be immobilized or freely rotating depending on the origin of the phospholipase A_2, and (3) the incorporation of phospholipase A_2 into micelles generally tends to reduce the overall rotation of the enzyme due to lipid binding and/or aggregation.

Registry No. Trp, 73-22-3; Phe, 63-91-2; phospholipase A_2, 9001-84-7.

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Intramolecular Electron and Proton Transfer in Proteins: CO_2^- Reduction of Riboflavin Binding Protein and Ribonuclease A

M. Faraggi, Jerald P. Steiner, and Michael H. Klapper*
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
Received November 15, 1984

ABSTRACT: The formate radical (CO_2^-) reacts with ribonuclease A to form the cystine disulfide radical as one of the products. CO_2^- reacts with the riboflavin binding protein of chicken egg white with the ultimate product being the neutral flavin semiquinone. Formation of the disulfide radical in ribonuclease is slower than the reaction between protein and CO_2^-; formation of the flavin semiquinone in the riboflavin binding protein is slower than the protein–CO_2^- reaction. We conclude for both proteins that CO_2^- must reduce an as yet unidentified group or groups, which in turn reduce(s) the disulfide of RNase or the flavin of riboflavin binding protein. This conclusion is supported in the case of ribonuclease by the observation of a transient, broad absorption band centered between 350 and 370 nm. The CO_2^--initiated reductions of the disulfide in ribonuclease and the flavin in the riboflavin binding protein are mixed first- and second-order processes. We propose that the transfer of an electron from the unknown intermediate(s) to the final product involves both inter- and intramolecular paths between groups that may not be in van der Waals contact. With the hydrated electron, in contrast to CO_2^-, as reductant of the riboflavin binding protein, the anionic semiquinone is observed as an intermediate. The anionic semiquinone is then rapidly protonated, yielding the stable neutral semiquinone. From the reaction kinetics and protein concentration dependence, we conclude that a group or groups on the protein donate(s) a proton to the anionic semiquinone by both inter- and intramolecular paths.

The proposal that electrons can migrate or transfer over long distances in proteins has been made a number of times, and for a variety of reasons [e.g., Winfield (1965), Grossweiner (1976), and Klapper & Faraggi (1979)]. Not only does the existence of such transfer have significance for the understanding of physiological redox reactions, but we have also suggested (Steiner et al., 1985) that the detection and study of protein intramolecular electron transfer may yield information about internal electrostatic properties of proteins. There have been a number of recent reports on electron transfer between groups that are not in van der Waals contact in small model systems [e.g., Prutz et al. (1981), Guar et al. (1983), Calcaterra et al. (1983), and Isied & Vassilian (1984)]. Two groups (Winkler et al., 1982; Isied et al., 1984)

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