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Rapid method to separate the domains of soybean lipoxygenase-1: identification of the interdomain interactions

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Abstract Lipoxygenase-1 (LOX1) from soybeans was cleaved with chymotrypsin (Ramachandran et al., 31 (1992) 7700–7706). The domains were separated on a Sephadex G-50 column by minimising domain interactions at pH 4.0. The molecular weight and apparent homogeneity of the domains were established by SDS-PAGE. The solution conformation of the 60 kDa and 30 kDa fragments was compared with that of native LOX1. 1-Anilino-8-naphthalene sulphonate (ANS) binding measurements confirmed the exposure of large hydrophobic residues on the surface of the 60 kDa due to separation of the domains. The monomeric nature of the 60 kDa fragment was confirmed by HPLC gel filtration. The increased number of binding sites and magnitude of binding constant suggested the involvement of extensive hydrophobic interactions between the two domains. The essential cofactor iron was with the C-terminal domain. The attempts to resolve and reconstitute the catalytic activity of isolated domains were not successful.

Key words: Lipoxygenase-1; Domain separation; Domain interaction; Surface hydrophobicity; Circular dichroism; Fluorescence

1. Introduction

Lipoxygenase-1 (LOX1) from soybeans consists of a single polypeptide chain with 839 amino acid residues and catalyses the hydroperoxidation of polyunsaturated fatty acids [1–3]. The X-ray crystallographic studies have shown that the enzyme consists of two domains: a small N-terminal β barrel domain consisting of 146 residues and a large principally α helical domain, consisting of 693 amino acid residues, and includes the catalytic iron binding site [1,4,5]. Based on the limited proteolysis of LOX1 by trypsin and chymotrypsin Ramachandran et al. [6] have reported that LOX1 consists of two domains: a roughly 30 kDa N-terminal domain and a roughly 60 kDa C-terminal domain [6,7]. The cleavage of LOX1 by chymotrypsin occurred at Phe-274 and Tyr-317, the resulting 'nicked enzyme' has fragments of 60 kDa and 30 kDa and a linker peptide of 43 amino acid residues. The fragments are tightly associated by strong non-covalent interactions. Thus, chymotryptic cleavage caused minimal changes in the native structure in a way that would affect catalysis. The proteolytic fragments are only partially resolved using size exclusion, hydrophobic interaction or ion exchange chromatography and the fragments do not dissociate in the absence of chaotropic agents.

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A comparison of the sequence of the various lipoxygenases has revealed that soybean enzymes are larger than their mammalian counterparts [8]. The mammalian enzymes share greater homology with the C-terminal region of the plant-derived lipoxygenases and lack the amino-terminal β barrel domain. At present the function of the N-terminus remains obscure and further all amino acids involved in the catalytic activity are located on the C-terminal domain of the enzyme, indicating that this domain of the enzyme could function independently of the N-terminal domain [5]. In an earlier paper we have reported the existence of kinetically stable intermediates during the course of unfolding by urea and guanidinium hydrochloride, which is attributed to the multidomain nature of the protein [9].

The present paper reports the separation method using a combination of molecular sieve chromatographic technique of chymotryptically cleaved LOX1 into two larger proteolytic domains of 30 kDa and 60 kDa proteins by minimising the domain interactions at pH 4.0. The solution conformation of the molecule was characterised using near-UV CD, far-UV CD and ANS binding studies and attempts were made to resolve and reconstitute the catalytic activity. The results obtained strongly suggest that the interdomain interactions could exert a critical role in maintaining the native folding of LOX1.

2. Materials and methods

2.1. Materials

LOX1 was isolated according to the method of Axelrod et al. [10] with some modifications after extraction, differential ammonium sulphate precipitation and dialysis. The protein loaded on DEAE-Sephadex A-50 column was eluted by a two-step linear gradient of–170 mM and then 170–230 mM sodium phosphate buffer (pH 6.8), the fractions containing LOX1 activity obtained in the second step gradient were concentrated by ammonium sulphate precipitation (0–70% saturation) and further purified on Sephadex G-75. The preparation had a specific activity of 220–240 μmol/min/mg of protein.

TLCK-treated chymotrypsin, phenylmethylsulphonyl fluoride (PMSF) and 1,8-ANS were purchased from Sigma. Linoleic acid was from Nucheck prep. MN, DEAE-Sephadex A-50, Sephadex G-75 and Sephadex G-50 were from Pharmacia.

2.2. Lipoxygenase-1 assay

The enzyme was assayed at pH 9.0 using 0.2 M sodium borate buffer according to the method of Axelrod et al. [10] by following the absorbance changes at 234 nm due to the formation of conjugated diene product. The protein concentration was estimated by absorbance measurements at 280 nm and using a value of $E^{1\%}_{1cm} = 14.0$, 17.94 and 7.26 for LOX1, 60 kDa fragment and 30 kDa fragment respectively; these values were obtained based on their absorption due to aromatic amino acid content. The effect of pH 4.0 on enzyme activity was measured after dialysing the enzyme at 4°C using 50 mM sodium acetate buffer for 12 h with three changes. The enzyme activity measured at pH 9.0 is expressed as percentage remaining activity at the end of 3 min compared with that of control.

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2.3. LOX1 proteolysis conditions and fragment separation

The chymotryptic digestion of LOX1 was performed according to Ramachandran et al. [6]. LOX1 and chymotrypsin (enzyme/substrate ratio = 0.1) in 0.1 M Tris-HCl, pH 8.0 at 25°C was incubated for 1 h. The reaction was terminated by adding 1 mM PMSF in methanol. The chymotrypsin was separated from the reaction mixture by gel permeation chromatography on Sephadex G-75 using the same buffer, the active fractions of 'nicked LOX1' were pooled and concentrated using an Amicon ultrafiltration cell with a 10 kDa cut-off membrane. Concentrated chymotrypsin-free proteolysed LOX1 was dialysed against 0.2 M acetate buffer pH 4.0 and chromatographed on a Sephadex G-50 column (2.5×90 cm) at 4°C. The column was pre-equilibrated and eluted with the same buffer. The lipoxygenase activity for nicked enzyme (dialysed against 0.2 M acetate buffer, pH 4.0), 60 kDa and 30 kDa fractions was checked at pH 9.0 as mentioned above, both fractions were mixed at pH 9.0 in a 1:1 molar ratio and kept for 10 h at 4°C and assayed for lipoxygenase activity.

2.4. Determination of molecular weight and iron content

SDS-polyacrylamide gel electrophoresis was carried out with 12% gels [11]. The samples were dialysed against sample buffer and heated on a boiling water bath for 90 s prior to loading on the gels. Proteins were stained using Coomassie brilliant blue.

The molecular weight of each protein was also determined at pH 4.0 (20 mM sodium dihydrogen phosphate, pH adjusted with phosphoric acid) using a Zorbax G-250 HPLC gel filtration column; the protein concentration was 2.8 mg/ml (15 μl was injected), the column was calibrated with standard proteins of known molecular mass and the assay was performed using a Shimadzu liquid chromatograph. The iron content in the 60 kDa fragment was estimated by using a PE 3110 Perkin-Elmer atomic absorption spectrophotometer as reported earlier [12].

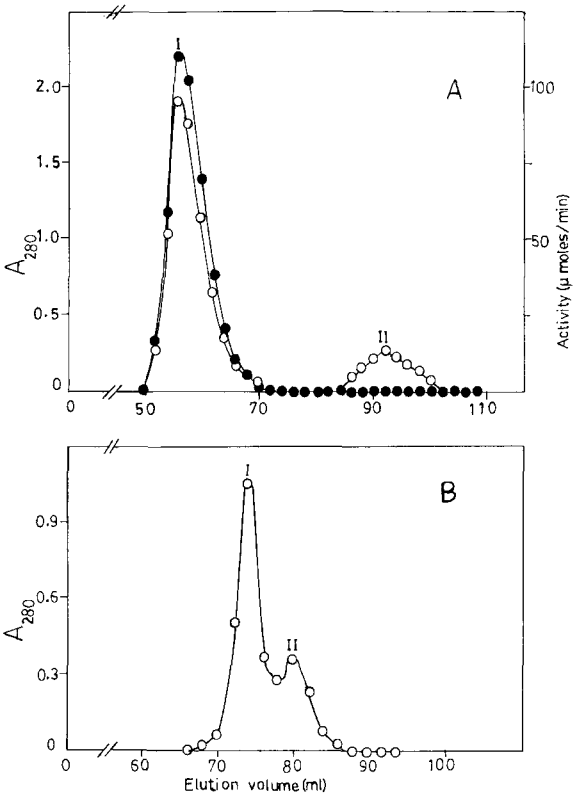

Fig. 1. A: Elution profile of proteolysed reaction mixture on Sephadex G-75. Eluted with 0.1 M Tris-HCl pH 8.0 at a flow rate of 0.4 ml/min, peak I is nicked LOX1 having LOX1 activity and peak II is PMSF-inactivated chymotrypsin. B: Molecular sieve chromatography on Sephadex G-50 column of nicked LOX1. Samples were eluted with 0.2 ml/min, SDS-PAGE determinations showed that peak I is 60 kDa and peak II is 30 kDa (○ is A_{280} and ● is LOX1 activity).

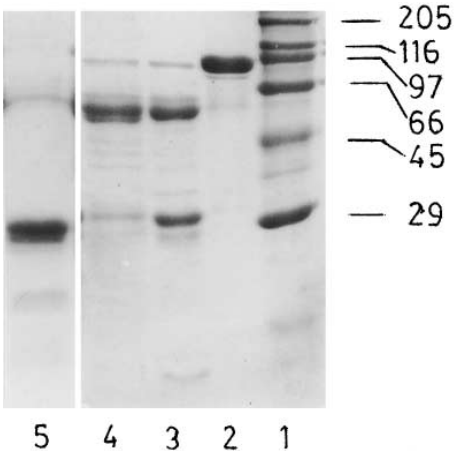

Fig. 2. SDS-PAGE analysis of the chymotryptically cleaved LOX1 fragments: molecular weight markers in kD (lane 1), native LOX1 (lane 2), nicked LOX1 (lane 3), 60 kDa (lane 4), and 30 kDa (lane 5).

2.5. Conformational analysis

Fluorescence emission spectra were recorded with a Shimadzu RF 5000 automatic recording spectrofluorophotometer equipped with a thermostatted cuvette holder. The excitation wavelength was 283 nm and the emission was recorded at 300–400 nm, circular dichroism spectra were recorded for LOX1, 60 kDa and 30 kDa in both far-UV and near-UV regions as described earlier [9]. All the spectroscopic measurements were made in 50 mM sodium acetate buffer, pH 4.0. The secondary structure content was estimated using the program CDPROT [13].

ANS binding studies were carried out as described by Cardamore and Puri [14] for LOX1, 60 kDa and 30 kDa in 50 mM sodium acetate buffer, pH 4.0. The excitation wavelength was 375 nm and the emission was recorded at 472 nm. The binding constants and the number of binding sites were determined and the surface hydrophobicity of each protein was obtained [15].

3. Results

3.1. Proteolytic fragment separation and characterisation

As previously reported LOX1 was incubated with 10% (w/w) chymotrypsin at pH 8.0 [6]. Based on activity and SDS-PAGE measurements, it was inferred that cleavage by chymotrypsin of LOX1 to 60 kDa and 30 kDa fragments was complete at the end of 60 min. To arrest further proteolysis of LOX1, chymotrypsin was inactivated by the addition of 1 mM PMSF, to separate the chymotrypsin from the nicked enzyme the reaction mixture was loaded onto a Sephadex G-75 column and analysed for the absence of chymotrypsin. The LOX1 activity was confined to the first peak and chymotrypsin to the second. The nicked enzyme eluted as a single symmetrical peak at the same position as native LOX1, indicating the tight association of domains at pH 8.0 (Fig. 1A). The nicked enzyme fractions were pooled and concentrated using the ultrafiltration system.

The nicked enzyme was taken to pH 4.0 by dialysing against 0.2 M sodium acetate buffer (pH 4.0). The dialysed enzyme was loaded on Sephadex G-50. The elution profile is shown in Fig. 1B. The fractions were analysed for the presence of 60 kDa and 30 kDa by SDS-PAGE (Fig. 2). The first peak contained 60 kDa and the second peak contained 30 kDa fragments (Fig. 1B).

As evident from the electrophoretic patterns, the first molecular sieve chromatography column separates the chymo-

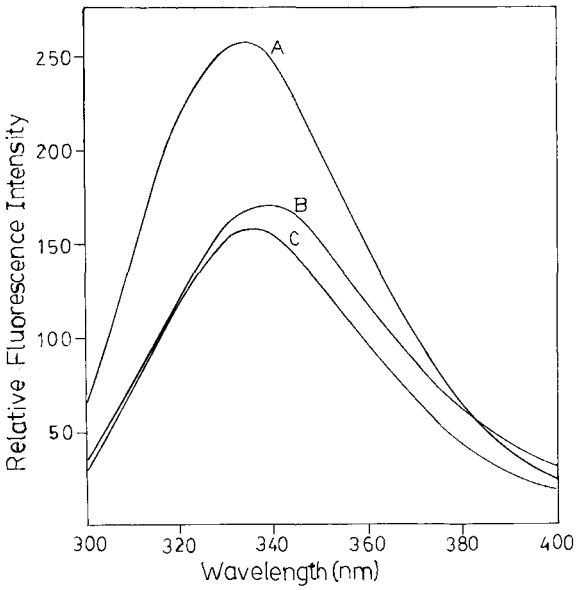

Fig. 3. Intrinsic fluorescence spectra of the native LOX1 (A), 60 kDa (B) and 30 kDa (C). Excitation at 280 nm.

trypsin from the nicked enzyme. Removal of chymotrypsin after inactivation with PMSF has to be accomplished at pH 8.0 only. Lowering the pH at that stage would reversibly activate chymotrypsin, leading to further degradation of nicked LOX1 [16]. Since some of the metalloproteins have a tendency to lose their cofactors at acidic pH to check whether the iron dissociates from the 60 kDa fragment at pH 4.0, the iron content was estimated using atomic absorption spectrophotometer, it showed the presence of iron in 60 kDa in a 1:1 molar ratio.

Although the nicked enzyme had 60% of the original activity, on dialysis from pH 8 to 4 it lost complete activity. However, LOX1 exposed to pH 4.0 by dialysis retained its complete activity. Fractions containing 30 kDa and 60 kDa displayed no catalytic activity. Mixing the resolved fragments in a 1:1 molar ratio for 10 h did not result in the recovery of catalytic activity under the conditions used for the LOX1 assay.

3.2. *Spectroscopic properties and conformational analysis*

All the conformational studies were carried out at pH 4.0, because the 60 kDa protein has a tendency to aggregate when the pH is raised from 4 to 9. The intrinsic fluorescence of the native LOX1, 60 kDa and 30 kDa fragments is shown in Fig. 3. When these proteins were excited at 283 nm, they showed emission maxima at 333 nm, 338 nm and 336 nm respectively. This is indicative of the exposure of aromatic chromophores to the solvent.

The CD bands of the protein in the near-UV range can be attributed to aromatic amino acids; upon separating the domains the tertiary structure has completely changed as reflected by the near-UV CD (Fig. 4A). LOX1 exhibits weak positive bands at 293, 288, 283, 274 and 266 nm. 60 kDa has increasingly negative bands at 310, 299, 284 and 279 nm. The secondary structure of the two fragments as indicated by the far-UV CD spectrum differs considerably (Fig. 4B and Table 1). The C-terminal 60 kDa fragment has more α helix than the native LOX1, the 30 kDa fragment has very little α helix and more of β structure. The X-ray diffraction data suggest that the N-terminal domain consists predominantly of β structure [1,5], all the major helical segments are in the C-terminal domain.

The binding of ANS to native LOX1, 60 kDa and 30 kDa is shown in Fig. 5A, with increasing ANS concentration up to 40 μM the relative fluorescence intensity increases and then levels off. The binding of ANS was analysed by Scatchard plot (Fig. 5B). The Scatchard plots were linear in all three cases, suggesting the presence of one set of equivalent binding sites. The association constant, the number of ANS binding sites and surface hydrophobicity are given in Table 2. The number of binding sites on LOX1 increased from one to nine in the case of 60 kDa protein, suggesting the exposure of a large number of hydrophobic residues; apparently there are a large number of ANS binding sites with greater affinity on 60 kDa which was also supported by a red shift in the fluorescence emission maximum (Fig. 3). The binding of ANS

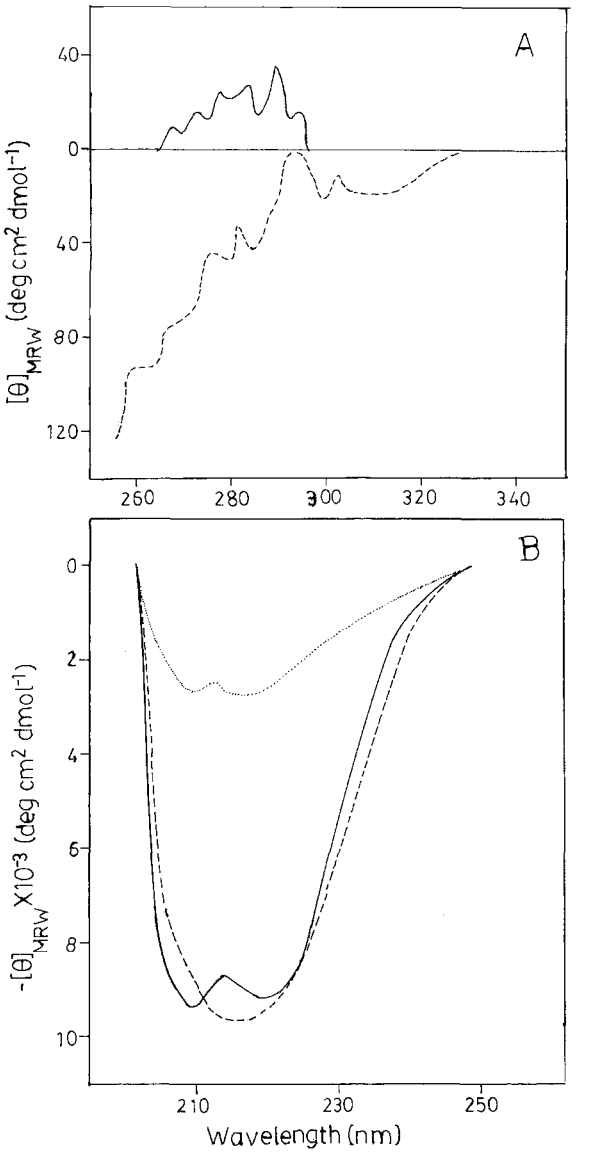

Fig. 4. Circular dichroism spectra. A: Near-UV CD. B: Far-UV CD. Native LOX1, solid line; 60 kDa, dashed line; 30 kDa, dotted line.

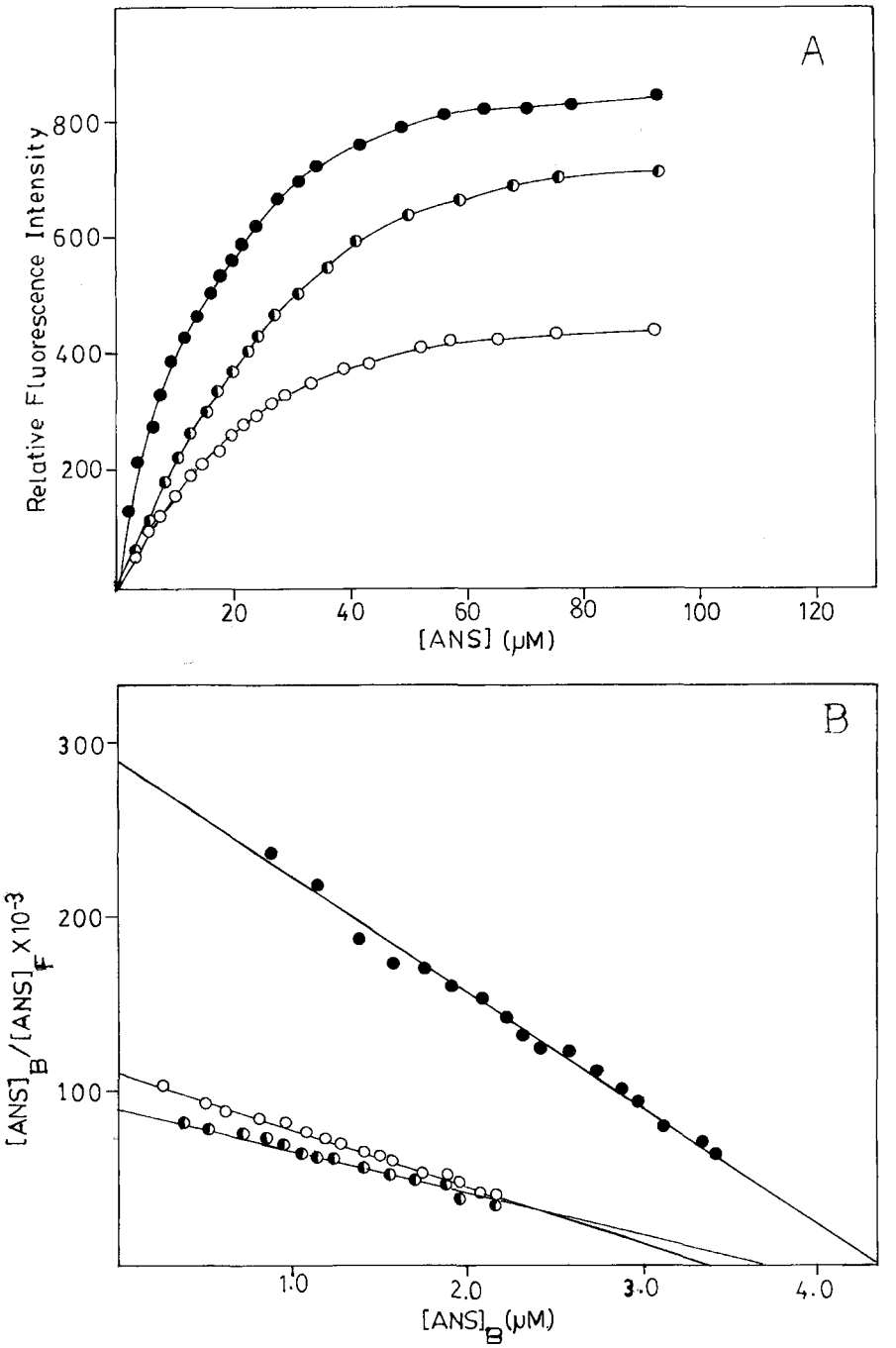

Fig. 5. Binding of ANS to native LOX1, 60 kDa and 30 kDa. A: Fluorescence titration of ANS bound to LOX1 (3.04 μM), 60 kDa (0.49 μM) and 30 kDa (3.84 μM). B: Scatchard plot for ANS binding (○ is LOX1, ● is 60 kDa and ◐ is 30 kDa).

to 30 kDa was very similar to that of LOX1 in terms of affinity and number of binding sites. The binding of ANS to LOX1 did not change significantly at pH 4 or 9 in terms of either affinity or the number of binding sites on the molecule. Although LOX1 has a hydrophobic cleft for binding the fatty acid at the active site, ANS did not inhibit LOX1 activity at any given concentration, indicating that ANS does not bind to the active site.

Further, to confirm that the increase in ANS binding sites in 60 kDa is not due to the aggregation, the monomeric nature of 60 kDa was established at pH 4.0 using HPLC size

Table 1
Computed percentage of α helix, β sheet, β bend and random structures

Sample	Proportion of			
	α helix	β sheet	β bend	random
Native LOX1	28	22	19	32
60 kDa fragment	31	24	22	23
30 kDa fragment	4	25	18	53

All estimations were performed using the program CDPROT which contains a method of Bolotina et al. [17], based on linear combination of reference spectra of proteins of known tertiary structure.

Table 2
Protein-ANS binding parameters and surface hydrophobicity

Protein	n	K_a (M^{-1})	SH
Native LOX1	1	23×10^3	110
60 kDa fragment	9	66×10^3	264
30 kDa fragment	1	28×10^3	ND

n = number of ANS binding sites per molecule of protein; K_a = binding constant for ANS with protein; SH = surface hydrophocity calculated according to method of Chaudhuri et al. [15]; ND = not determined.

exclusion chromatography on a Zorbax G-250 column, which gave an estimated molecular mass of 60 kDa.

4. Discussion

Using limited proteolysis Ramachandran et al. have reported that the chymotryptically clipped LOX1 separated under denaturing conditions [6,7]. Separation by size exclusion, hydrophobic interaction or ion exchange chromatographic techniques was not successful because of the extensive interdomain interactions. The nicked LOX1 holds both the domains very tightly at pH 8.0. Our studies show that the decrease of pH from 8.0 to 4.0 helps to break the domain interactions, thereby causing the decoupling of domains. The native LOX1 undergoes reversible structural changes in the region of pH 9.0 to 4.0 (Sudharshan, Srinivasulu and Appu Rao, unpublished data). At pH 4.0, there is decoupling of domains of the nicked enzyme. Thus, it could be separated by molecular sieve chromatography on Sephadex G-50 column. Limited chymotryptic cleavage of LOX1 occurs at two sites, between Phe-274 and Asp-275 and Tyr-317 and Arg-318. Taking account of the X-ray and limited proteolysis studies, it appears that the chymotrypsin-cleaved bonds resides one in α2 helix (Phe-274 and Asp-275) and the other in β10 sheet (Tyr-317 and Arg-318), although there are several aromatic amino acids flanking these sites, only two potential sites are susceptible to chymotryptic cleavage, which suggests only the above two sites are exposed to protease attack.

Analysis of the iron content of the C-terminal fragment confirmed the presence of iron in it. The decrease in pH from 8 to 4 decouples the domains, which had no effect on the iron linkages. The native LOX1 when taken from pH 9 to 4 retains all its activity reversibly. Although the catalytic centre was present in the 60 kDa, it did not exhibit LOX1 activity. Reconstitution experiments with resolved fragments did not result in the recovery of catalytic activity. These observations indicated that while limited proteolysis divided LOX1 into two large fragments, a proper coupling of domains or interactions between two domains is necessary for maintenance of the native three-dimensional structure to express the catalytic activity. The tertiary structure of the C-terminal domain differs considerably from that of LOX1 as reflected in the near-UV CD, the secondary structures of two domains are substantially different, the C-terminal 60 kDa fragment is rich in α helix and the N-terminal 30 kDa fragment has predominantly β structure: the 30 kDa has an α helical content of 4% in solution versus 19% in the crystal structure, the 60 kDa fragment has an α helical content of 31% in solution versus 47% in crystal. This would seem to indicate that both 30 kDa and 60 kDa fragments are partially unfolded when isolated from the LOX1 molecule. Thus chymotrypsin cleaves LOX1 into two structural units and consists mostly of its secondary structures, which it possessed when it was linked in the native enzyme.

The ANS binding measurements suggested that the 60 kDa fragment has nine ANS binding sites; this could be due to the exposure of larger number of hydrophobic residues which were involved in interdomain interactions or getting exposed to the solvent once the domains are separated. Further, 30 kDa also has one hydrophobic binding site. Thus the presence of a hydrophobic binding site in both the domains suggests an extensive hydrophobic interactions which could contribute significantly to the strong interdomain interactions. However, even the intact LOX1 molecule also has one hydrophobic binding site, which could be associated with the 60 kDa domain. Because, taken together with ANS not inhibiting LOX1 activity and one each ANS binding site in LOX1 and 30 kDa, it indicates that apart from the hydrophobic fatty acid binding site there is another hydrophobic binding site in LOX1, which could have a critical role in lipoxygenase catalysis.

In conclusion, our results suggest that in the LOX1 molecule there are extensive interdomain interactions which could exert a critical role in maintaining the native folding to express the catalytic activity. Our results also provide an easy method to generate large amounts of 60 kDa and 30 kDa fragments, retaining most of their secondary structure, which could be the object of further structural and functional studies.

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