Catalysis Sensitive Conformational Changes in Soybean Lipoxygenase Revealed by Limited Proteolysis and Monoclonal Antibody Experiments†

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ABSTRACT: Soybean lipoxygenases catalyze lipid hydroperoxidation of polyunsaturated fatty acids. Putative ligand mediated conformational changes in soybean lipoxygenase 3 (L3) were studied by a combination of limited proteolysis and a series of monoclonal antibodies that recognize discontinuous epitopes and alter catalysis (inhibition and activation). Trypsin cleaved L3 (97 kDa) into C-terminal 60 kDa and N-terminal 37 kDa fragments. The 37 kDa fragment was obtained from a 38 kDa fragment formed initially. Using protein footprinting, the epitopes of the antibodies were mapped to the 37 kDa fragment. Proteolysis in the presence of a substrate analog inhibitor, oleic acid, generated the 60 and the 38 kDa fragments only. No further proteolysis of the 38 kDa fragment was seen even after prolonged incubation. This was not a detergent effect since the altered proteolysis pattern was not obtained in the presence of SDS or Tween 20. Binding of a monoclonal antibody to L3 in the presence of oleic acid was substantially reduced providing additional evidence for a conformational change induced by the oleic acid−lipoxygenase interaction. These observations are interpreted using the recently solved three-dimensional structure of L3. It is apparent that while the protein is composed of a small N-terminal β-barrel domain and a large principally α-helical C-terminal domain, proteolysis does not take place at a linking region between the two domains. The proteolysis result makes it clear that the smaller domain is connected across the entire length of the larger domain to a narrow, tongue-like projection that extends into the vicinity of the entrance to the proposed substrate binding channel. It is proposed that conformational changes take place upon oleic acid binding which are transmitted through the protein and alter structural features in the N-terminal fragment that are sensitive to proteolysis and antibody binding. This is the first direct evidence for conformational changes related to catalysis in lipoxygenase.

Lipoxygenase plays a fundamental role in polyunsaturated fatty acid metabolism in plants and animals. The enzyme is responsible for the inauguration of the biosynthesis of leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids from arachidonic acid in numerous mammalian cells (Samuelsson et al., 1987). In plants the substrates for hydroperoxidation are linoleic and linolenic acids, and the products are intermediates in the biosynthesis of compounds with growth regulatory as well as pest resistance properties (Siedow, 1991). Soybean lipoxygenase-1 has been available in a highly purified form since 1947 (Theorell et al., 1947), yet the details of the structure and mechanism of action of the enzyme are only now beginning to emerge.

The plant lipoxygenases are all large (M_r 95 000) monomeric polypeptides with a single non-heme iron cofactor (Pistorius & Axelrod, 1974). The primary sequences for lipoxygenases began to become available from the sequencing of their cDNAs in 1987 (Shibata et al., 1987,1988; Yenofsky et al., 1988). Now sequences from a number of plant and animal sources are available. Sequence comparisons have revealed certain highly conserved regions in the proteins from diverse sources (Feiters et al., 1990). For example, conserved histidine residues have been proposed to comprise the non-heme iron binding site, and this has been confirmed in recent site-directed mutagenesis studies (Steczko & Axelrod, 1992). The nature of the iron site and its role in catalysis have also been investigated using a number of spectroscopic techniques. It is apparent that the iron atom participates in catalysis (Funk et al., 1990), but how the protein involves the cofactor in the reaction is far from clear. Two solid state structures for soybean lipoxygenase-1 have been published recently (Boyington et al., 1993; Minor et al., 1993). The accounts differ significantly on the composition of the iron binding site. The three-dimensional structure of the L3 isoenzyme solved recently is similar to that of L1 (Skrzypczak-Jankun et al., 1994). In contrast to the numerous investigations probing the nature of the non-heme iron site, the conformational states of lipoxygenases in solution have not been extensively studied and require further investigation for a detailed understanding of catalysis by this enzyme.

Using limited proteolysis experiments, we recently demonstrated that lipoxygenase-1 consists minimally of two domains, a roughly 30 kDa N-terminal region and a roughly 60 kDa C-terminal domain (Ramachandran, 1992). The conserved histidines thought to be involved in iron binding

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are all located in the larger proteolytic fragment, and this fragment was covalently labeled by treatment with phenylhydrazine. These observations place the iron binding site and by implication the active site in the larger, C-terminal domain. Our conclusions have been borne out by the three-dimensional structures obtained from X-ray crystallography.

We recently reported the production of a battery of monoclonal antibodies against two isoenzymes of soybean lipoxygenase (Wheelock et al., 1991). Antibodies selective for the isoenzymes and the different domains were obtained. Some of these antibodies were capable of activating or inhibiting L3 catalysis. Here the techniques of limited proteolysis and protein footprinting with monoclonal antibodies were employed to reveal catalysis sensitive conformational changes in the protein. The data presented indicate that specific conformational changes accompany ligand binding.

MATERIALS AND METHODS

Materials. Lipoxygenase 3 (L3) was obtained from soybeans *cv.* Provar by extraction, differential ammonium sulfate precipitation, dialysis, and chromatofocusing (Funk et al., 1986). TPCK-trypsin, soybean trypsin inhibitor, and oleic acid were obtained from Sigma. Anti-mouse IgG-Sepharose was from Organon-Teknika. Gel electrophoresis supplies were obtained from Bio-Rad. All other reagents and solvents were of the highest purity available. Monoclonal antibodies and ascites fluid were prepared as described previously (Wheelock et al., 1991). Ascites fluid was stored in liquid nitrogen until use.

Limited Proteolysis of Lipoxygenase 3. Trypsin digestion was carried out by treating L3 (0.5 mL, 0.25 mg, 0.2 M Tris-HCl, pH 7.5) with trypsin (0.050 mL, 0.025 mg, 0.2 M Tris-HCl, pH 7.5) at 1 °C for the times shown. Reactions were terminated by combining an aliquot of the digestion solution (0.05 mL) with a solution of soybean trypsin inhibitor (0.005 mL, 0.0025 mg, 0.2 M Tris-HCl, pH 7.5). Proteolysis in the presence of oleic acid was done by incubating L3 (0.5 mL, 0.25 mg, 0.2 M Tris-HCl, pH 7.5) at 1 °C for 5 min followed by serial additions of oleic acid [0.02 mL, 10% (v/v), methanol] and trypsin (0.05 mL, 0.025 mg, 0.2 M Tris-HCl, pH 7.5). Reactions were terminated as described above. Proteolysis was carried out in the presence of the detergents SDS and Tween 20 using the same procedure.

Immunochemical Procedures. Immunoblot and immunoprecipitation experiments were carried out as described previously (Wheelock et al., 1991). For protein footprinting L3 (0.5 mL, 0.050 mg, 0.1 M Tris-HCl, pH 7) was incubated with 0.003 mL of ascites fluid containing the monoclonal antibody at 4 °C for 90 min, added to anti-mouse IgG Sepharose beads, and incubated for 1 h with gentle shaking at 4 °C. The beads were washed repeatedly with 150 mM NaCl, 1% Triton, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5. The beads were then treated with Trypsin (0.5 mL, 0.04 mg, 0.1 M Tris-HCl, pH 7.5) at 37 °C for the time specified. The beads were pelleted and washed thoroughly and finally treated with 0.075 mL of Laemmli sample buffer. Antibody binding to L3 in the presence of oleic acid was carried out by incubating L3 (0.05 mg, 0.5 mL, 0.1 M Tris-HCl, pH 7.4) with 0.003 mL of ascites fluid containing the antibody in the presence of oleic acid [0.004 mL, 1% (v/v), methanol] for 1 h on ice followed by addition to 0.2 mL of anti-mouse IgG Sepharose beads. After gentle shaking for 30 min at 4 °C, the beads were pelleted, washed repeatedly, and treated with 0.05 mL of sample buffer for SDS gel electrophoresis. SDS−polyacrylamide gel electrophoresis determinations were carried out with 12% gels (Laemmli, 1970). The samples were prepared for electrophoresis by treatment in boiling water for 90 s in a 1:1 ratio with sample buffer.

Peptide Mapping and Sequencing. Peptide mapping was carried out using *Straphylococcus aureus* V8 protease (Cleveland et al., 1977). Sequencing of proteolytic fragments was carried out following gel electrophoresis or reversed phase HPLC. The proteins were transferred to Problot membrane (Applied Biosystems) and visualized by Coomassie brilliant blue R-250 staining. Appropriate bands were cut and automated Edman sequencing was done on an Applied Biosystems Model 477A protein sequencer. HPLC samples were applied directly to the sequencer.

Three-Dimensional Structure Determination. The three-dimensional structure was solved using the method of molecular replacement (Skrzypczak-Jankun et al., 1994). The model was constructed using X-PLOR (Brünger et al., 1987). The ribbon diagram was generated by the program MOLSCRIPT (Kraulis, 1991).

RESULTS

Limited Proteolysis Experiments. Preliminary screening revealed that antibodies capable of influencing catalysis were obtained to only one lipoxygenase isoenzyme. In the past we have referred to this isoenzyme as P4 (the first of four lipoxygenases to emerge from a chromatofocusing column during the isolation from soybean seeds, *cv.* Provar). From protein and cDNA sequencing studies we have determined that this isoenzyme most closely resembles the lipoxygenase referred to as L3 by others (Kramer et al., 1994). The experiments reported here were carried out on this isoenzyme. When the L3 isoenzyme of lipoxygenase was exposed to trypsin under nondenaturing conditions, two major fragments migrating with relative molecular masses of 59 and 37 kDa were obtained as illustrated in Figure 1 (top). This was similar to the result reported for lipoxygenase-1 earlier (Ramachandran, 1992). As was the case for lipoxygenase-1, cleavage of the L3 isoenzyme took place with no change in specific activity. For example, samples from nine time points over the course of a typical digestion experiment had 1.08 ± 0.05 times the specific activity of an untreated control. The two major fragments were isolated by reversed phase HPLC and subjected to automated Edman sequence determination. The larger fragment was subjected to N-terminal sequence determination through 19 cycles. The sequence matched exactly with that reported for L3 (Yenofsky et al., 1988) for positions 337−355 beginning at Thr-337. The 37 kDa fragment was sequenced through 14 cycles. The sequence matched exactly with positions 9−22 in the published L3 sequence starting with Gly-9. These results demonstrate, as was found for lipoxygenase-1, that the enzyme consists of a smaller N-terminal domain and a larger C-terminal domain. The time course for the limited proteolysis of the two isoenzymes by trypsin was, however, different. Lipoxygenase-1 digestion proceeded rapidly to two proteolytically stable fragments. By contrast, in the proteolysis of L3, formation of the smaller, 37 kDa fragment

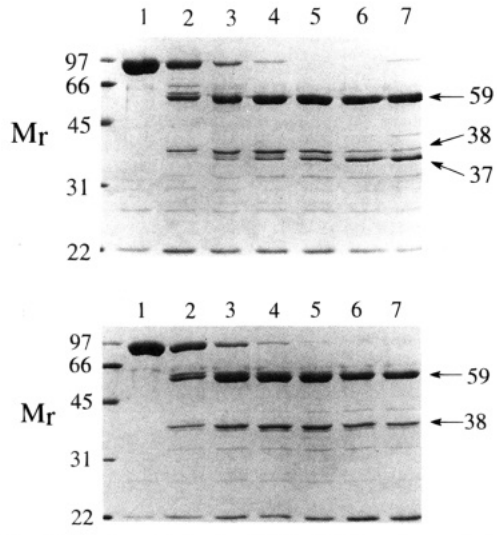

FIGURE 1: Limited proteolysis of L3 with trypsin carried out at 1 °C in 0.2 M Tris-HCl, pH 7.5. L3 was mixed with trypsin and samples were withdrawn at 0, 6, 15, 25, 45, 100, and 120 min and analyzed on a 12.5% SDS−polyacrylamide gel (lanes 1−7, respectively). The band at the bottom of the gel is the soybean trypsin inhibitor used to stop the reaction. Molecular weight marker are indicated. (Top) L3 alone. (Bottom) L3 preincubated with 2500× molar excess of oleic acid.

proceeded through an intermediate polypeptide appearing at a relative molecular mass of 38 kDa. No sequence was obtained from the 38 kDa band presumably due to a blocked N-terminus (Shibata et al., 1988). The conversion of the band migrating at 38 kDa to the stable 37 kDa polypeptide was completely prevented by incubation of the enzyme with oleic acid. This is demonstrated in Figure 1 (bottom). Proteolysis was done at 1 °C with 2500× molar excess of oleic acid to ensure saturation of the L3 with oleic acid at all times. At this ratio, the concentration of oleic acid was 12.5 mM. Protection was also observed at 250× (1.25 mM) and 100× (0.5 mM) molar excess of oleic acid (data not shown). It has been reported that oleic acid acts as a competitive inhibitor of lipoxygenase (Holman, 1947) and also protects it from inactivation by phenylhydrazine, a putative mechanism-based inhibitor (Gibian & Singh, 1986). The effect of oleic acid was specific. Incubations with SDS or Tween-20 did not prevent the 38 to 37 kDa conversion (data not shown). These observations indicate that trypic cleavage sites which are exposed to proteolysis in the absence of oleic acid become inaccessible to digestion in its presence.

Epitope Mapping Studies. As previously reported (Wheelock et al., 1991), only a small fraction of the monoclonal antibodies, 6 of the original 96, recognized continuous epitopes (linear sequences) on the P4/L3 isoenzyme revealed by Western blot determinations. This is not surprising since the intact native lipoxygenase was used as the original antigen. Another set of five antibodies which bound to discontinuous epitopes and by inference the native enzyme were capable of influencing catalysis. The epitopes of these antibodies were all localized to the smaller N-terminal fragment by protein footprinting, a procedure initially developed by Sheshbaradaran and Payne (1988). Lipoxygenase isoenzyme L3 was incubated with one of the catalytically activating or inhibiting antibodies followed by addition of anti-mouse IgG attached to agarose beads to form a ternary complex. The complex was treated with trypsin for different incubation times, and the cleaved peptides not

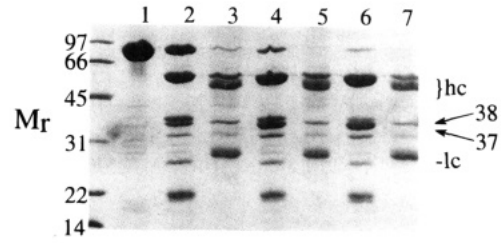

FIGURE 2: Protein footprinting of L3 with monoclonal antibodies recognizing discontinuous epitopes. L3 was incubated with ascites fluid containing one of the antibodies (8653-2 #46), bound to anti-mouse Sepharose and proteolyzed with trypsin or cleaved with trypsin in the absence of the antibody−bead complex for 30 min (lanes 2 and 3), 60 min (lanes 4 and 5), and 90 min (lanes 6 and 7). Samples were resolved on 12.5% SDS−polyacrylamide gel. Lane 1, L3; lanes 2, 4, and 6, L3 with trypsin in the absence of the antibody; lanes 3, 5, and 7, protein footprinting of L3 with monoclonal antibody. Molecular weight markers are indicated at left. The positions of the antibody heavy chains (hc) and light chains (lc) are indicated.

retained bythe complex were removed by thorough washing. The retained protein fragments were analyzed by SDS−polyacrylamide gel electrophoresis (Figure 2). The results are shown for antibody 8653−2 #46. Similar results were also obtained with the other four antibodies. Samples from proteolysis of L3 in the absence of antibody at different time points are shown for comparison. Only polypeptide with a relative molecular mass of approximately 37 kDa was detected in the protein footprinting experiments at all time points. The density of the bands from the protein footprinting experiment and the time course in the absence of antibody are not directly comparable because of the widely differing conditions by which they were generated. The bands at higher relative molecular masses in samples from protein footprinting were conclusively identified as the heavy chains from the antibodies in Western blotting experiments, i.e., the bands were immunoreactive with anti-mouse IgG but not with antibodies to the larger lipoxygenase fragment. This indicated that the larger 59 kDa fragment from L3 was washed away from the ternary complex of the 37 kDa fragment of L3, the antibody, and the anti-mouse IgG agarose. Thus it appeared from these results that the epitopes recognized by both the activating and inhibitory antibodies were located in the smaller N-terminal fragment. This assignment of epitope was confirmed by sequence analysis of the 37 kDa band obtained from the protein footprinting experiment. No sequence was obtained from the intact 37 kDa. This was not expected since the 37 kDa band from trypsin digestion alone produced a sequence beginning with Gly-9. The 37 kDa band from the protein footprinting experiment was therefore subjected to further proteolysis by *S. aureus* V8 protease in polyacrylamide (Cleveland et al., 1977) followed by resolution by SDS−polyacrylamide gel electrophoresis. This procedure produced a predominant polypeptide with a relative molecular mass of 19 kDa that was transferred to a nitrocellulose membrane and subjected to automated Edman sequence determination. A clear sequence extending 11 residues and beginning with Gly-9 in the overall sequence of isoenzyme L3 was obtained. This identified the 37 kDa band retained in the antibody complex in the protein footprinting experiment as the N-terminal fragment. It is not clear why the 37 kDa band alone did not provide sequence data in this instance, since the trypsin site at Arg-8 was evidently already cleaved in that polypeptide.

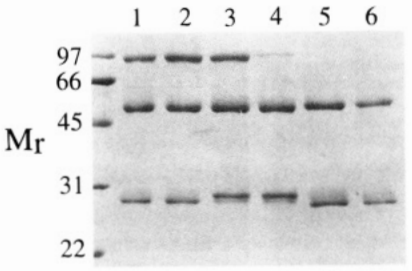

FIGURE 3: Binding of monoclonal antibodies recognizing discontinuous epitopes to L3 in the presence of oleic acid. L3 was immunoprecipitated with the ascites fluid of the monoclonal antibodies in the presence or absence of oleic acid. Lanes 1, 3, and 5, without oleic acid; lanes 2, 4, and 6, with oleic acid. Lanes 1 and 2, L3 with antibody 8653−9 #33; lanes 3 and 4, L3 with antibody 8653−7 #33; lane 5, L3 with unrelated antibody (control); and lane 6 pure antibody (control). Molecular weight markers are indicated at left. Bands around 50 and 30 kDa are due to the antibody heavy and light chains, respectively.

Oleic Acid Affects Antibody Binding. The limited proteolysis experiments indicated that the conformation of the protein was altered in the presence of oleic acid relative to the unclipped enzyme. In some cases the affinity of antibodies for discontinuous epitopes can be altered by conformational changes in proteins. Therefore we sought to utilize the antibodies that recognize discontinuous epitopes in order to provide further evidence that a conformational change does occur in lipoxygenase when the inhibitor binds. The enzyme was incubated with two inhibitory antibodies (8653−7 #33 and 8653−9 #33) either in the presence or absence of oleic acid. The complexes were immunoprecipitated with anti-mouse IgG agarose beads. The immune complexes were washed and the retained proteins were analyzed by SDS−polyacrylamide gel electrophoresis. The results of this experiment are illustrated in Figure 3. The ability of antibody 8653−9 #33 to bind to lipoxygenase P4 was clearly not affected by the presence of oleic acid, while oleic acid substantially reduced binding of antibody 8653−7 #33 under the conditions of this experiment. The interpretation of this observation is that the discontinuous epitope recognized by the 8653−7 #33 is altered by a conformational change in the protein resulting from oleic acid binding.

DISCUSSION

Limited proteolysis of lipoxygenase-3 with trypsin divided the protein into two parts, a larger C-terminal and a smaller N-terminal fragment. As was found for lipoxygenase-1, the clipped enzyme retained catalytic activity, and the two fragments did not dissociate in the absence of chaotropic agents. Cleavage took place at the same point as was previously reported for lipoxygenase-1 when the two sequences were aligned for maximum sequence identity: after Arg-318 in L1 and after Arg-336 in L3. While the outcome of limited proteolysis was the same in both instances, the time course was found to be significantly different for the two isoenzymes. The smaller fragment produced from L3 by trypsin digestion was obtained in a clearly resolved two-step process. It was apparent that there was a 38 kDa intermediate in the formation of the proteolytically stable 37 kDa fragment. Cleavage at Arg-8 of L3 would reduce the mass of the smaller fragment by 877 mass units not including the unknown N-terminal blocking group which could account for the formation of the 37 kDa fragment. There are also trypsin sites after Lys-332 and Lys-324 in the L3 sequence. Cleavage at one of these sites after the initial break after Arg-336 would reduce the mass of the smaller fragment by an additional 563 or 1429 mass units, respectively. The range of values for the difference in the two smaller fragments is on the order of 800−2400 mass units (38 200 ± 700 amu minus 36 600 ± 300 amu = 1600 ± 800 amu). Therefore, we cannot distinguish between cleavage only at the N-terminal site and cleavage at an internal site as well on the basis of the available data.

The step-wise process observed for limited proteolysis by trypsin was completely blocked by prior incubation of L3 with the inhibitor of lipoxygenase catalysis, oleic acid. The effect was specific to oleic acid and was observed in the concentration range expected for ligand binding. The K_m value for L3 acting on linoleic acid as a substrate has been reported as 0.34 mM. In one study of the inhibitory effect of oleic acid, a 50% effect was found at a ratio of inhibitor to substrate of 10:1 (Holman, 1947). That would be 3.4 mM oleic acid for a half-maximal effect at a substrate concentration near K_m. The effect of oleic acid on proteolysis is seen in the concentration range of 0.5−12.5 mM. While these concentrations are within the range of the critical micelle concentration reported for oleic acid (0.72 mM), they are in the same range as for those used in inhibition studies and do not therefore represent a vast excess of ligand. The presence of oleic acid apparently changes the conformation of the protein in a way that renders the formerly susceptible site after Arg-8 (and possibly Lys-324 or Lys-332 as well) inaccessible to trypsin digestion. We assume that the initial digestion by trypsin has a negligible effect on the conformation since the clipped enzyme retains full catalytic activity.

Changes in protein conformation upon ligand binding have been well documented. Changes involving extensive movements of elements of protein secondary structure have been characterized. For example, when triglyceride lipase binds the inhibitor diethyl *p*-nitrophenylphosphate, a "lid" consisting of 15 amino acids incorporating a short α-helix moves by more than 12 Å when compared to atomic positions in the native enzyme (Derewenda et al., 1992). Conformational flexibility in proteins in response to ligand binding extends to the movement of entire domains relative to each other. For example, on the basis of X-ray crystallographic data, when adenylate kinase binds AMP, C_α atoms in one domain translocate by as much as 32 Å relative to their positions in the native enzyme (Gerstein et al., 1993). A conformational change in the solution structure of a protein can lead to an altered susceptibility to proteolysis. In a recent example, the cellular retinoid binding proteins were found to be more susceptible to proteolysis in the apo or ligand free form (Jamison et al., 1994). Ligand binding reduced proteolysis in this family of proteins. A conformational change involving the movement of a helical cap for the ligand binding site was proposed to account for the differences in structure. Therefore, there are numerous examples of conformational changes relevant to catalytic activity and its regulation taking place in enzymes. The limited proteolysis findings reported here indicate that conformational flexibility may also be an intrinsic feature of lipoxygenase catalysis.

Conformational flexibility appears to be the most satisfactory explanation for the effects of certain antibodies on lipoxygenase catalysis as well. Only antibodies recognizing discontinuous, i.e., conformation-dependent epitopes, were

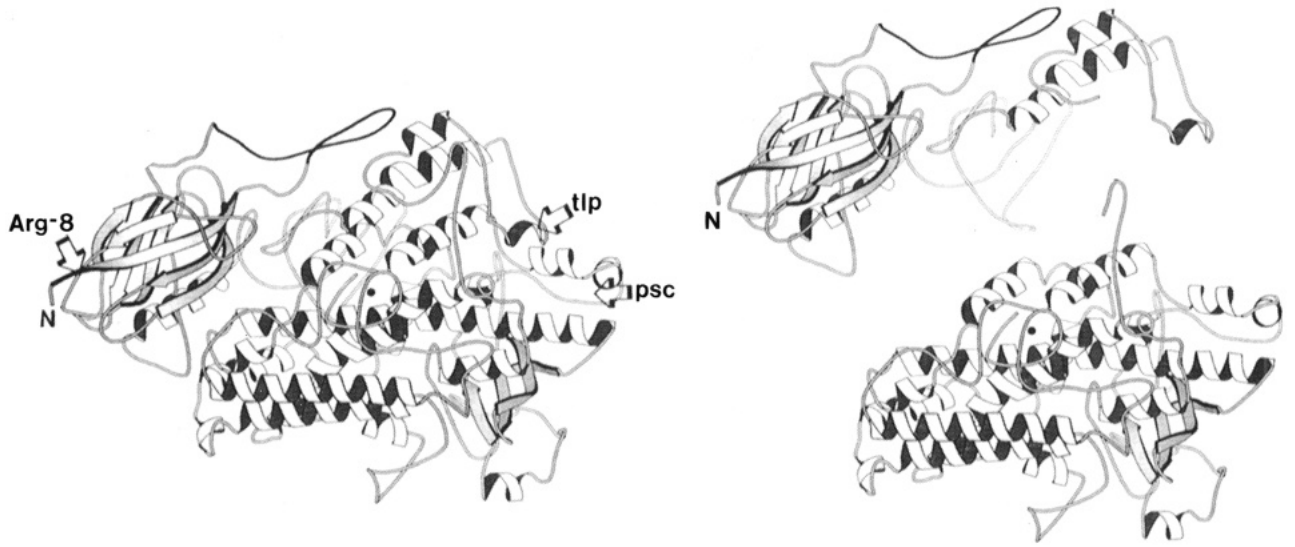

FIGURE 4: (Left) Ribbon drawing of L3 lipoxygenase prepared with the program MOLSCRIPT (Kraulis, 1991). The N-terminal is labeled, and a black dot indicates the position of the iron atom. The positions of the proposed substrate channel (psc), the tongue-like projection (tlp), and the N-terminal trypsin cleavage site (Arg-8) are indicated. (Right) The two trypsin cleavage fragments have been artificially moved apart from each other to indicate their interaction surfaces. The black coil marks a loop disordered in X-ray structure.

capable of influencing catalysis. Using the protein footprinting technique, the epitopes of these antibodies were conclusively localized to the smaller N-terminal fragment. It appears that, by binding to the smaller fragment, some of the antibodies stabilize a conformation that is activated for catalysis, while others stabilize a conformation that is less active (Wheelock et al., 1991). The relationship between antibody binding and conformation was also found in the effect of oleic acid on the antigen−antibody interaction. Inhibitor binding drastically reduced the affinity of one of the antibodies to L3 while preserving the binding of another antibody. It is apparent from these findings that the relationship between antibody binding and catalysis is a reciprocal one. Antibody binding affects catalysis (activation and inhibition), and ligand (inhibitor) binding affects the conformation of the epitopes. Altered affinity for monoclonal antibodies upon ligand binding had been used previously as evidence for conformational changes in enzymes. For example, in a series of monoclonal antibodies to the β_2 subunit of tryptophan synthase, some antibodies had substantially higher affinity for the protein in the presence of ligand, while the affinity of other antibodies was not affected (Djavadi-Ohaniance et al., 1986). These observations were used to suggest that conformational changes in specific regions of the enzyme were associated with ligand binding. It has been demonstrated that antibody binding can have an effect on the rate of exchange of protons from a protein antigen to the bulk solvent at locations quite remote from the complementarity determining region (Benjamin et al., 1992). This result has been interpreted as evidence for an antibody induced long-range conformational change in the protein antigen. It appears that lipoxygenase is capable of antibody induced long-range conformational alterations that have a direct consequence in terms of catalytic activity.

A model for the three-dimensional structure for the L3 isoenzyme, which has been constructed from X-ray diffraction data (Skrzypczak-Junkun et al., 1994), reveals how the results of monoclonal antibody and limited proteolysis experiments can be reconciled with the domain organizationof the protein. In Figure 4 two representations of the structural model show where limited proteolysis takes place in the context of the overall folding pattern of the protein. The lipoxygenase-3 isoenzyme clearly consists of two compact domains as was found for lipoxygenase-1 (Boyington et al., 1993). A small N-terminal domain consisting primarily of a β-barrel motif is attached through a linking sequence to a larger C-terminal, principally α-helical domain. Strikingly, however, limited proteolysis does not take place in the linking region between the two domains as is common for multidomain proteins even though there are as many as 12 potential trypsin sites in the span. Instead, the cleavage reaction produces an N-terminal fragment that includes the β-barrel motif and roughly 150 additional residues that make continuous contact with one of the hemispherical surfaces of the C-terminal fragment. The additional segments of the chain adopt both α-helical and random coil conformations. Interestingly, a portion of the chain forms a long, narrow tongue-like projection with a short helix at the end that extends into the vicinity of the entrance of one of the two channels leading from the surface of the protein to the nonheme iron site. It has been proposed that the channel approached by the tongue-like feature is the substrate binding site.

The structural features of the L3 lipoxygenase provide a basis for understanding the biochemical results. The N-terminal fragment and the sites for limited proteolysis by trypsin are connected rather directly to an element of the structure that could be expected to be conformationally sensitive to the presence of substrate or inhibitor molecules at the proposed active site. The tongue-like projection is reminiscent of the hinged helix lid features seen in the structures of the cellular retinoid-binding proteins and triglyceride lipase. Oleic acid binding could produce a conformational change transmitted through the tongue-like feature and the protein framework to the N-terminus which renders the trypsin cleavage site after Arg-8 inaccessible. Such a conformational change could also be expected to alter specific discontinuous epitopes reducing antibody affinity. Likewise, antibody binding to the N-terminal fragment could influence the conformation of the protein at the access point

for the active site by the same connectedness in the protein folding. It is not clear, however, if the epitopes are present in the small N-terminal domain or in the roughly 150 amino acid extension of the N-terminal fragment that is part of the larger C-terminal domain. The epitopes and the trypsin cleavage site can not be very close together. In cases where specific structural information is available on antibody–antigen interactions, binding has been found to take place over a significant surface area of a protein antigen (>700 Å^2) (Sheriff et al., 1987). The possibility that the antibody combining sites are close to the point of trypsin cleavage would require simultaneous interaction of L3 with both the antibody and the protease in the case of the protein footprinting experiment. The antibodies and trypsin could conceivably be interacting with the 150 amino acid extension on opposite sides of the L3 molecule. If the epitopes are located in the smaller N-terminal domain, then the reciprocal effects of the antibodies and ligands is a long range one involving interactions between the two domains.

The comparison of the sequences of the various lipoxygenases has revealed that the soybean enzyme is larger than its mammalian counterparts (Fleming et al., 1989). The mammalian enzymes share greater homology with the C-terminal portion of the soybean enzyme, which corresponds to the larger, iron bearing trypsin fragment. The activity of the 5-lipoxygenase is stimulated by a small membrane associated activating protein, FLAP (5-lipoxygenase activating protein) (Miller et al., 1990). The two proteins apparently interact in a way that enhances catalysis. The activity of 5-lipoxygenase from HL-60 cells is also stimulated by a heat stable factor in human serum (Steinhibler, 1993). While the species responsible for this regulatory effect has not been identified, preliminary results indicate that it is probably a protein because it is susceptible to proteases and is not retained by 10 000 MWCO dialysis membranes. Therefore, the activity of 5-lipoxygenase is stimulated by at least two other proteins. It is tempting to speculate that the small N-terminal trypsin fragment of the soybean lipoxygenases plays the role of the mammalian activating proteins by establishing and maintaining the appropriate conformation of the enzyme for the expression of catalytic activity.

Evidence for conformational changes in lipoxygenase-1 in response to changes in the microenvironment were reported very recently (Pourplanche et al., 1994). Subtle changes in the conformation induced by the water-soluble additives, sucrose and sorbitol, were detected in the Raman spectrum. The global secondary structure of the protein was not altered. The presence of sorbitol or sucrose during catalysis changed the regioselectivity of product formation. Therefore, a change in the microenvironment was correlated with a change in the conformation and the regiochemical outcome of catalysis. Our observations with limited proteolysis and monoclonal antibodies confirm and extend these findings. The observations reported here establish that soybean lipoxygenases also experience ligand- and antibody-induced conformational changes.

REFERENCES

Benjamin, D. C., Williams, D. C., Jr., Smith-Gill, S. J., & Rule, G. S. (1992) *Biochemistry 31*, 9539–9545.

Boyington, J. C., Gaffney, B. J., & Amzel, L. M. (1993) *Science 260*, 1482–1486.

Brünger, A. T., Kuriyan, J., & Karplus, M. (1987) *Science 235*, 458–460.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Derewenda, U., Brzozowski, A. M., Lawson, D. M., & Derewenda, Z. S. (1992) *Biochemistry 31*, 1532–1541.

Djavadi-Ohaniance, L., Friguet, B., & Goldberg, M. E. (1986) *Biochemistry 25*, 2502–2508.

Feiters, M. C., Boelens, H., Veldink, G. A., Vliegenthart, J. F. G., Navaratnam, S., Allen, J. C., Nolting, H.-F., & Hermes, C. (1990) *Recl. Trav. Chim. Pays-Bas 109*, 133–146.

Fleming, J., Thiele, B. J., Chester, J., O'Prey, J., Janetzki, S., Aitken, A., Anton, I. A., Rapoport, S. M., & Harrison, P. R. (1989) *Gene 79*, 181–188.

Funk, M. O., Carroll, R. T., Thompson, J. F., & Dunham, W. R. (1986) *Plant Physiol. 82*, 1139–1144.

Funk, M. O., Carroll, R. T., Thompson, J. F., Sands, R. H., & Dunham, W. R. (1990) *J. Am. Chem. Soc. 112*, 5375–5376.

Gerstein, M., Schulz, G., & Chothia, C. (1993) *J. Mol. Biol. 229*, 494–501.

Gibian, M. J., & Singh, K. (1986) *Biochem. Biophys. Acta 878*, 79–92.

Holman, R. T. (1947) *Arch. Biochem. Biophys. 15*, 403–413.

Jamison, R. S., Newcomer, M. E., & Ong, D. E. (1994) *Biochemistry 33*, 2873–2879.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946–950.

Kramer, J. A., Johnson, K. R., Dunham, W. R., Sans, R. H., & Funk, M. O. (1994) *Biochemistry 33*, 15017–15022.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Miller, D. K., Gillard, J. W., Vickers, P. J., Sadowski, S., Leveille, C., Mancini, J. A., Charleson, P., Dixon, R. A. F., Ford-Hutchinson, A. W., Fortin, R., Gauthier, J. Y., Rodkey, J., Rosen, R., Rouzer, C. A., Sigal, I., Strader, C. D., & Evans, J. F. (1990) *Nature 343*, 278–281.

Minor, W., Steczko, J., Bolin, J. T., Otwinowski, Z., & Axelrod, B. (1993) *Biochemistry 32*, 6320–6323.

Pistorius, E. K., & Axelrod, B. J. (1974) *J. Biol. Chem. 249*, 3183–3186.

Pourplanche, C., Lambert, C., Berjot, M., Marx, J., Chopard, C., Alix, A. J. P., & Larreta-Garde, V. (1994) *J. Biol. Chem. 269*, 31585–31591.

Ramachandran, S., Carroll, R. T., Dunham, W. R., & Funk, M. O. (1992) *Biochemistry 31*, 7700–7706.

Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8075–8079.

Samuelsson, B., Dahlen, S.-E., Lindgren, J. A., Rouzer, C. A., & Serhan, C. N. (1987) *Science 237*, 65–84.

Sheshberadaran, H., & Payne, L. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1–5.

Shibata, D., Steczko, J., Dixon, J. E., Hermodson, M., Yazdanparast, C., & Axelrod, B. (1987) *J. Biol. Chem. 262*, 10080–10085.

Shibata, D., Steczko, J., Dixon, J. E., Andrews, P. C., Hermodson, M., & Axelrod, B. (1988) *J. Biol. Chem. 263*, 6816–6821.

Siedow, J. N. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*, 145–188.

Skrzypczak-Jankun, E., Funk, M. O., Boyington, J. C., & Amzel, L. M. (1994) in *The 9th Organic Crystal Chemistry Symposium* (Katrusiak, A., & Jones, D. W., Eds.) p 112, Poznan'-Rydzyna, Poland.

Steczko, J., & Axelrod, R. (1992) *Biochem. Biophys. Res. Commun. 186*, 686–689.

Steinhibler, D., Rådmark, O., & Samuelsson, B. (1993) *Biochem. Biophys. Res. Commun. 193*, 1083–1090.

Theorell, H., Holman, R. T., & Akeson, A. (1947) *Acta Chem. Scand. 1*, 571–576.

Wheelock, M. J., Richards, T. J., Carroll, R. T., & Funk, M. O. (1991) *Arch. Biochem. Biophys. 288*, 578–583.

Yenofsky, R. L., Fine, M., & Liu, C. (1988) *Mol. Gen. Genet. 211*, 215–222.

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