Bengal Gram Lipoxygenase: Fluorescence Quenching Study of the Interaction of Linoleic Acid and 13- and 9-Hydroperoxylinoleic Acids with the Two Forms of the Enzyme

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The intrinsic protein fluorescence of the two forms of lipoxygenases from Bengal gram has been characterized. The fluorescence is dominated by emission from tryptophan residues in a hydrophobic environment. The substrate linoleic acid and the reaction products 13- and 9-hydroperoxylinoleic acids quenched the intrinsic protein fluorescence equally for two forms of the enzyme without lag period. From the fluorescence quenching measurements, the association constant (K) and the free energy change for the interaction have been calculated. The two forms of the enzyme differ in their affinity to the substrate. The $\Delta G°$ value for the interaction of substrate/products was calculated to be −5.0 kcal/mol, suggesting that the interaction is a weak one. Spectroscopic measurements do not indicate a large conformational change in the enzyme due to the binding of these molecules.

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the oxidation of linoleic acid and other polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene system. The primary product of this oxygenation reaction is a cis,trans-conjugated dienoic hydroperoxy derivative. The enzyme from soybean is well characterized and has been shown to exist in multiple forms with distinct kinetic and immunological properties (Axelrod et al., 1981). The lipoxygenase from Bengal gram (*Cicer arietinum*) extract has been resolved into two active forms which have shown to be kinetically distinct with respect to the products they generate from linoleic acid (Brothakur and Ramadoss, 1986). The two forms also differ in the positional specificity for the oxygenation of linoleic acid (Borthakur et al., 1987). There are many reports in the literature regarding the intrinsic fluorescence intensity of lipoxygenase and its quenching by substrate (Finazzi-Agro et al., 1973; Shechter and Grossman, 1983; Macias et al., 1988). Here we report on the interaction of the two forms of the enzyme from Bengal gram with linoleic acid and 13- and 9-hydroperoxides as studied by fluorescence spectroscopy.

MATERIALS AND METHODS

Materials and Chemicals. Seeds of Bengal gram were obtained from the Seed Corporation of India, Mysore, India. Linoleic acid was obtained from Nu Check Prep, Elysian, MN. DEAE-Sephadex was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxyapatite was prepared as described by Bernardi (1971).

Preparation of the Substrates. A stock solution of 10 mM sodium linoleate containing Tween 20 was prepared as described by Axelrod et al. (1981).

Isolation of the Enzyme. Lipoxygenases 1 and 2 from the Bengal gram extract were purified as described earlier (Borthakur et al., 1988). The specific activities of the enzyme preparations used in these studies were 95 and 67, respectively, for Bengal gram lipoxygenases 1 and 2.

The enzyme was assayed either by following the oxygen consumption on a Gilson Oxygraph or by the appearance of conjugated diene absorbing at 234 nm with a Beckman Model-26 spectrophotometer. One unit of activity was defined as the utilization of 1 μmol of substrate or the formation of 1 μmol of product per minute under assay conditions.

Protein was determined from $E^{1\%}_{280\,nm}$ which was found to be 12.5 for both lipoxygenases 1 and 2.

Isolation of 13-L- and 9-D-Hydroperoxylinoleic Acids. The 13-L- and 9-D-hydroperoxyoctadecadienoic acids (13-LooH and 9-LooH) were prepared by aerobic incubation of linoleic acid with soybean lipoxygenase 1 (Garssen et al., 1971) and the potato enzyme at pH 6.5 (Galliard and Philips, 1971), respectively, as described earlier (Borthakur et al., 1987).

Fluorescence. The excitation and emission spectra of the enzyme samples were taken on an Aminco-Bowman spectrophotofluorometer. The excitation and emission wavelengths were calibrated with quinine sulfate. The emission spectra were recorded after excitation of the sample at 285 nm for 15 s. Protein samples of less than 0.1 absorbance at the excitation wavelength were used to avoid inner filter effects. The relative fluorescence intensities of the enzyme samples and the effects of the addition of the substrate and products were measured at 330 nm. Proper blanks were run by using tryptophan solutions having the same absorbance as that of the protein at 280 nm. All the measurements were made with protein solutions in 50 mM phosphate buffer, pH 6.5, that is the optimum pH for both lipoxygenases 1 and 2. The temperature of the samples was maintained at 25 °C by circulating water around the cell. The quenching of relative fluorescence intensity by substrate and products was analyzed in terms of binding of substrate and products using established procedures (Appu Rao and Cann, 1981; Appu Rao et al., 1989).

To get an estimate of the stoichiometry of binding, the data were analyzed by the equation of Ikeda and Hamaguchi (1969)

$$\log [Q/(Q_{max} - Q)] = \log K + n \log C \quad (1)$$

where Q is the corrected fluorescence intensity, Q_{max} is the maximal fluorescence quenching, C is the molar constituent concentration of substrate/product, and n is the binding stoichiometry. Q_{max} was determined by extrapolation of a double-reciprocal plot of Q versus C to the intercept.

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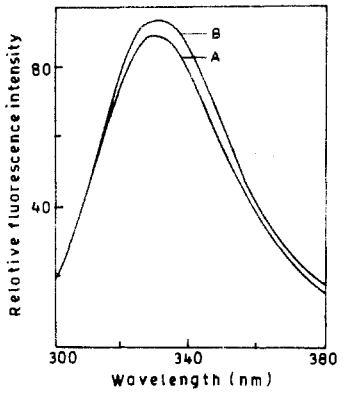

Figure 1. Fluorescence emission spectra of Bengal gram lipoxygenase (excitation at 285 nm). (A) Lipoxygenase 1, 0.087 mg/mL; (B) lipoxygenase 2, 0.096 mg/mL.

RESULTS AND DISCUSSION

The fluorescence emission spectra of lipoxygenases 1 and 2 after excitation at 285 nm are shown in Figure 1. Both forms of the enzyme showed intrinsic fluorescence emission maximum around 330 nm. The addition of the substrate linoleic acid and the reaction products, 13- and 9-hydroperoxide, resulted in a consistent decrease in the enzyme fluorescence emission spectra without a shift in the emission maximum at 330 nm. However, the addition of Tween 20 alone, at a concentration similar to that of the final reaction mixture, did not result in any quenching. The emission wavelength of the two forms of enzyme suggests that the tryptophans are in a nonpolar environment (Shifrin et al., 1971). The shape of the fluorescence spectra is not affected in the quenched samples, which could mean either that the tryptophans are equivalent in terms of polarity or that differently solvated tryptophans have much lower quantum yields. These fluorescence quenching measurements do not point to large conformational changes in enzymes induced by these molecules.

To follow the interaction of linoleic acid and the reaction products, 13- and 9-hydroperoxide, with the two forms of the enzyme, the quenching of the fluorescence intensity at 330 nm was followed as a function of concentration of these molecules (Figure 2). We could not test higher concentrations of the ligand because of turbidity development. As can be seen from Figure 2 that at 100 μM concentration of linoleic acid, the fluorescence intensity of lipoxygenases 1 and 2 at 330 nm was quenched to the extent of 35% and 70%, respectively (Figure 2A). The net quench in fluorescence intensity due to the addition of 13-hydroperoxide (Figure 2B) and 9-hydroperoxide (Figure 2C) was similar with both of the enzymes.

The plot of $1/Q$ versus the reciprocal of substrate/product concentration was linear. The Q_{max} values were calculated from the intercept of the plot and are given in Table I. In all cases Q_{max} was found to be more than 100%, the physical significance of which is not clear. By use of this value of Q_{max} logarithmic plots were constructed for both the enzymes, with either substrate or products; the slope of the line was 1.0 ± 0.07, indicating that one molecule of linoleic acid/13-LOOH/9-LOOH was bound per mole of enzyme. Mass-action plots are shown in Figure 3. From the slope of the line the equilibrium association constant was calculated (Table I).

The values of the association constant were found to be different for lipoxygenases 1 and 2 for their interaction with linoleic acid (Table I), while with 13- and 9-hydroperoxide these values are similar for both enzymes. The

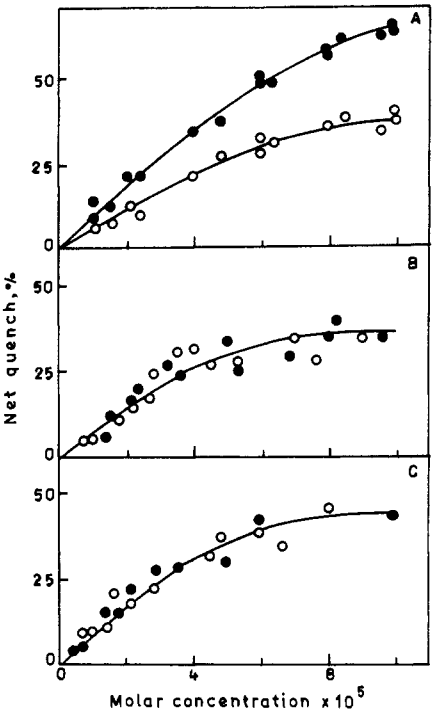

Figure 2. Variation of relative fluorescence intensity of lipoxygenase 1 (○) and lipoxygenase 2 (●) as a function of ligand concentration. (A) Linoleic acid; (B) 13-hydroperoxylinoleic acid; (C) 9-hydroperoxylinoleic acid.

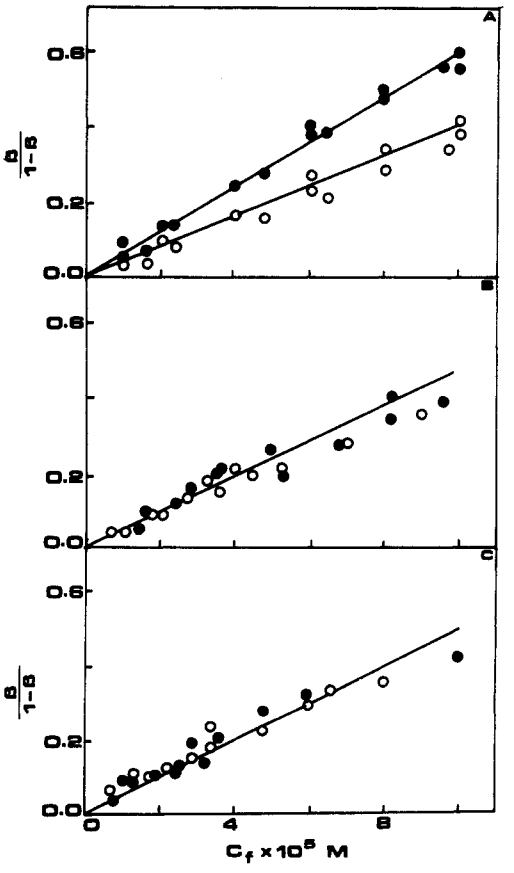

Figure 3. Mass-action plot of $\beta/(1-\beta)$ against C_f for lipoxygenase 1 (○) and lipoxygenase 2 (●). (A) Linoleic acid; (B) 13-hydroperoxylinoleic acid; (C) 9-hydroperoxylinoleic acid.

free energy change for the interaction was derived by using the equation $\Delta G° = -RT \ln k$. The $\Delta G°$ values thus obtained for the interaction of linoleic acid, 13-hydroperoxide, and 9-hydroperoxide with lipoxygenases 1 and 2

Table I. Values of Maximal Quench (Q_{max}) of the Fluorescence of BGL1 and BGL2 by Linoleic Acid and 13- and 9-Hydroperoxide and the Association Constant (K) for the Interaction

	max quenching (Q_{max}), %		assoc constant (K), mol L^{-1}	
ligand	BGL1	BGL2	BGL1	BGL2
linoleic acid	147	172	4.5×10^3	5.8×10^3
13-hydroperoxide	166	166	3.6×10^3	3.8×10^3
9-hydroperoxide	170	170	4.9×10^3	4.9×10^3

were around -5.0 ± 0.1 kcal/mol. This low value is probably indicative of weak interaction of the ligands with both forms of the enzyme.

Fluorescence studies on soybean lipoxygenase 1 by Finazzi-Agro et al. (1973) also suggested that tryptophan residues are in a nonpolar environment. It should be noted that soybean L-1 is optimally active at pH 9.0, while the Bengal gram enzymes have pH optimum below neutrality. The presence of tryptophan in the active site has been shown for both soybean (Klein et al., 1985) and Bengal gram enzymes (Borthakur et al., 1988). These findings perhaps are suggestive of different ionizable groups in the active-site regions of the soybean and Bengal gram enzymes. Yet the environments around the active-site tryptophan residues in these enzymes appear similar.

We have shown earlier that Bengal gram lipoxygenases 1 and 2 differ with respect to the products they generate from linoleic acid (Borthakur and Ramadoss, 1986). Liopyxgenase 1 converted linoleic acid exclusively to linoleic acid hydroperoxide, while lipoxygenase 2 generated ketodienes in addition to the hydroperoxide. The two forms of the enzyme also differed in the positional specificity for the oxygenation of linoleic acid (Borthakur et al., 1987). Lipoxygenase 1 produced both the 9- and the 13-isomer in a ratio of 79:21. The two forms were also shown to have dissimilar tertiary structure as was evident from nonidentical near-UV CD spectra, although they possessed a similar secondary structure (Borthakur et al., 1988). The structural distinction between lipoxygenases 1 and 2 is also evident from the differential ability of the substrate, linoleic acid, to quench the fluorescence under aerobic condition. In the case of soybean lipoxygenase 1, Finazzi-Agro et al. (1973) have reported that the fluorescence emission intensity is quenched by linoleic acid and the 13-hydroperoxide. The quenching by the substrate was shown to be due to the formation of the product as there was no quenching by linoleic acid under anaerobic condition. Although the results on the fluorescence quenching of the Bengal gram enzymes are similar to those observed for soybean L-1, the concentrations of the substrate or the product required to bring about maximum quenching differed considerably. A maximum quenching for the soybean enzyme was obtained with a stoichiometric amount of 13-hydroperoxide, while the Bengal gram enzymes required a severalfold excess of linoleic acid and the hydroperoxides. The K_m values reported for Bengal gram lipoxygenases 1 and 2 were 7 and 80 μM, respectively (Borthakur and Ramadoss, 1986). It may be mentioned that at the enzyme concentration of 1 μM in these studies the molar equivalent of the substrate is far below the K_m value for linoleic acid and hence perhaps unable to saturate all of the binding site on the enzyme. It is interesting to note that 9-hydroperoxylinoleic acid also quenched the fluorescence of Bengal gram lipoxygenase 1, whose reaction products are almost exclusively the 13-isomer. Even the association constant for the binding of the 9-isomer is similar to that of the 13-isomer.

It has been observed that in the kinetics of the soybean L-1 catalyzed reaction there is a lag, and this lag can be abolished effectively by the addition of the product of the reaction (Haining and Axelrod, 1958; Smith and Lands, 1972). From the analysis of the kinetic data, Egmond et al. (1976) showed that there is a mutually exclusive site for the interaction of substrate and product on the soybean L-1 beside the active site. The Bengal gram enzymes did not show any lag in the kinetics of product formation (Borthakur et al., 1988). Moreover, the fluorescence data indicated the presence of only one binding site either for the substrate or for the product. The binding constant for the interaction of substrate with soybean lipoxygenase 1 calculated from kinetic data is of the order of 10^5 mol L^{-1} (Jyothrimayi, unpublished results). This is at least an order of magnitude higher than that of the Bengal gram enzymes reported here. Thus, the Bengal gram enzymes differ from soybean lipoxygenase 1 not only in their pH optima but also in their affinity for the substrate.

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