FLUORESCENCE PERTURBATION IN SOYBEAN LIPOXYGENASE-1

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1. Introduction

Recently, we observed [1] changes in the absorption spectrum and in the fluorescence behaviour of soybean lipoxygenase-1 evoked by 13-L_S-hydroperoxy-linoleic acid, the product of the oxygenation of linoleic acid catalyzed by this enzyme. It was proposed [1], that these changes are related to the conversion of iron in the native enzyme into the ferric state. Upon anaerobic addition of an excess of linoleic acid the spectral characteristics of the native enzyme reappeared. The fluorescence quenching was attributed to a non-radiative energy transfer since a new absorption band appeared in the wavelength region of tryptophan emission. Previously [2], it has been shown that removal of oxygen causes a fluorescence decrease without an apparent formation of a new absorption band.

The mechanism of this fluorescence was investigated in more detail by using external perturbing agents like KI, imidazole, DMSO, KCNO, $CsCl_2$ and NaN_3 since the method of solute perturbation of fluorescence can give important information about the interactions between the fluorescent species and the bulk of the solution [3,4].

2. Materials and methods

Soybean lipoxygenase-1 was isolated according to Finazzi Agrò et al. [2].

Absorbance, fluorescence and CD-spectroscopy were carried out as described before [2].

Anaerobiosis was obtained by four cycles of vacuum-argon. The distance R_0 for 50% energy transfer was calculated from the following equation [5]:

$$R_0^6 = \frac{8.8 \times 10^{-25}\, \Phi_D \cdot J_{AD'} \cdot K^2}{n^4} \qquad (1)$$

wherein:

$$J_{AD'} = \int_0^\infty \frac{\epsilon_A(\nu) \cdot F_D(\nu)}{\nu^4}\, d\nu$$

$\epsilon_A(\nu)$ = decadic molar extinction coefficient of the acceptor; $F_D(\nu)$ = the spectral distribution of the donor emission normalized to unity,
ν = frequency
K^2 = dipole-dipole orientation factor,
n = refractive index of the intervening medium,
Φ = quantum yield of the donor.

For lipoxygenase-1 $J_{AD'}$ was calculated by numerical integration of linear wavelength values of the absorption intensity and of fluorescence. Φ was taken 0.1 [2]. A value of 2/3 for K^2 was chosen (cf. Discussion).

3. Results

3.1. *Fluorescence quenching by iodide*

As shown in fig.1 the quenching of lipoxygenase fluorescence by KI is proportional to the amount of

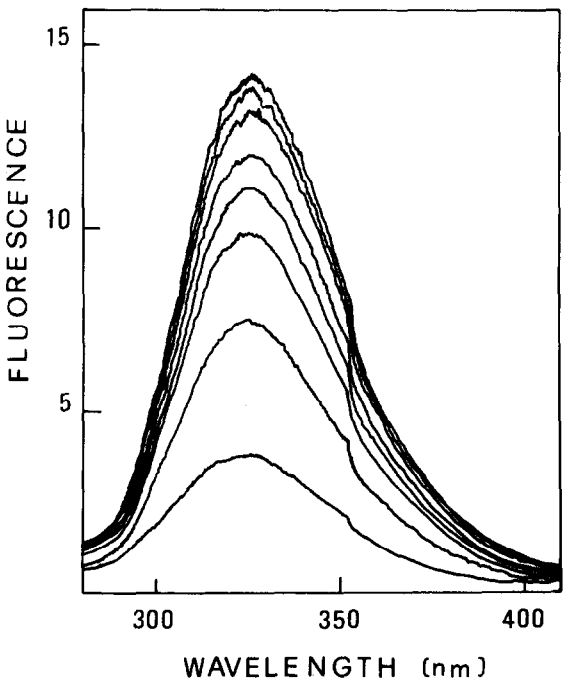

Fig.1. Quenching of lipoxygenase fluorescence by KI. From the top: 1.3×10^{-7} M lipoxygenase in 0.1 M carbonate buffer pH 9.4; and after addition of 10^{-2} M KI; 3×10^{-2} KI 10^{-1} M KI; 1.97×10^{-1} M KI; 3.66×10^{-1} M KI; 9.1×10^{-1} M KI, 2.17 M KI.

iodide added. KCl, up to a concentration of 2 M, did not affect the fluorescence.

Fig.2 and fig.3 show the effect of I^- on F_o/F in relation to pH and the presence of O_2. Evidently, lowering of the pH enhances the effect of I^- (1 M): at pH 9.4 50% quenching and at pH 5.3 65% quenching is observed.

Removal of O_2 gives a much higher quenching than without previous removal of oxygen (fig.3) (re-

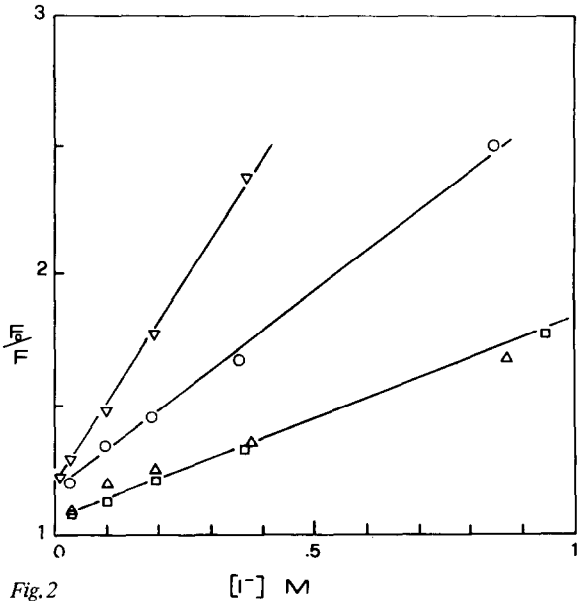

Fig.2

Fig.2. Stern–Volmer plots of iodide quenching effect on lipoxygenase in air. (□–□–□) in 0.1 M carbonate buffer pH 9.4; (△–△–△) in 0.1 M phosphate buffer pH 7.3; (○–○–○) in 0.1 M acetate buffer pH 5.3; (▽–▽–▽) in 0.1 M acetate buffer pH 3.1. Lipoxygenase concentration: 1.8×10^{-6} M in all the samples.

Fig.3. Stern–Volmer plots of iodide quenching effect on lipoxygenase after four evacuation cycles. (■–■–■) in 0.1 M carbonate buffer pH 9.4; (▲–▲–▲) in 0.1 M phosphate buffer pH 7.3; (●–●–●) in 0.1 M acetate buffer pH 5.3. Lipoxygenase concentration: 1.8×10^{-6} M in all the samples.

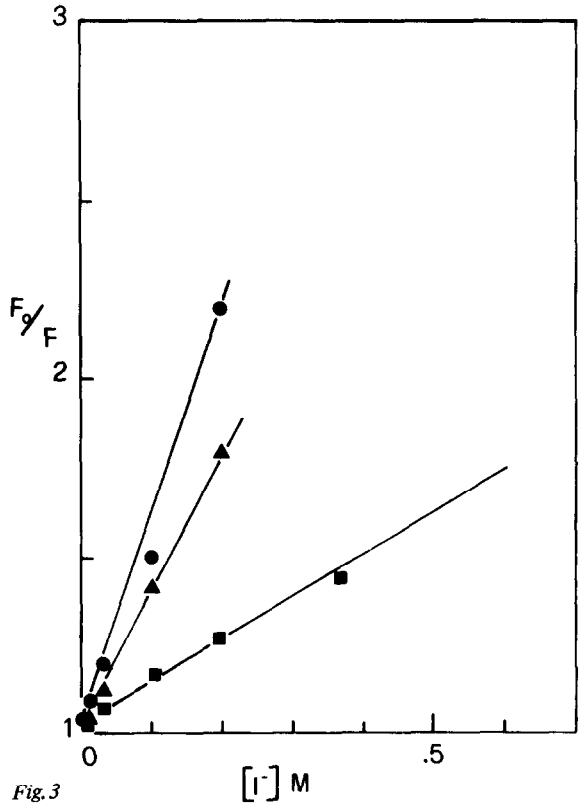

Fig.3

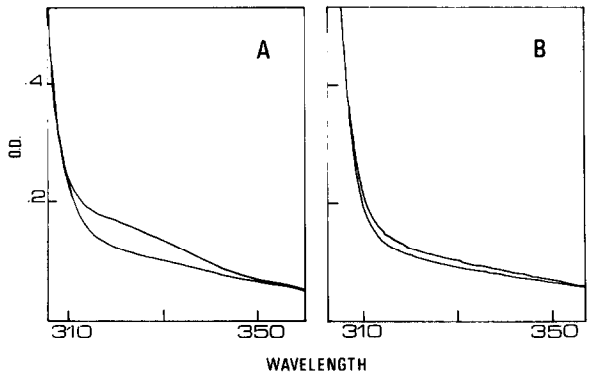

Fig.4. Absorption spectra of lipoxygenase in the presence of KI. (A) 1) 1.3×10^{-4} M lipoxygenase in 0.1 M acetate buffer pH 5.3. 2) After addition of 1 M KI. (B) 1) 1.3×10^{-4} M lipoxygenase in 0.1 M carbonate buffer pH 9.4. 2) After addition of 1 M KI.

oxygenation of the protein is an extremely slow process [2]).

Fig.4 shows the absorbance changes at these pH values induced by I^- (1 M).

At pH 5.3 the absorption band, which overlaps the emission of the tryptophan residues in lipoxygenase-1, has an extinction coefficient of about 300 $1\ M^{-1} \cdot cm^{-1}$ at 325 nm, while at pH 9.4 the extinction coefficient was found to be about 100 $1\ M^{-1} \cdot cm^{-1}$.

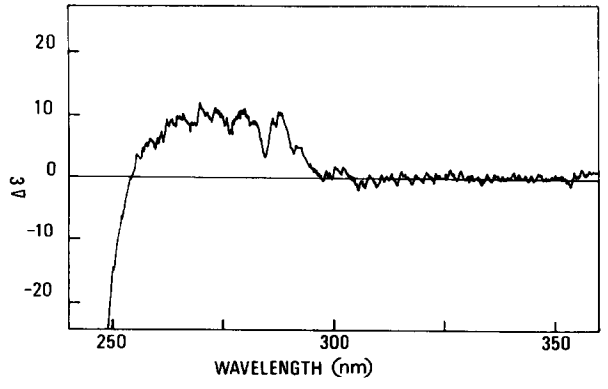

Fig.5. Near ultraviolet CD spectrum of lipoxygenase. 10^{-5} M lipoxygenase dissolved in 0.1 M carbonate buffer pH 9.4. Optical pathlength: 1 cm.

3.2. *Fluorescence quenching by other agents*

Imidazole (1 M) and DMSO (2.6 M) did not cause any fluorescence quenching, while KCNO (1 M) and $CsCl_2$ (1 M) gave about 10% quenching at pH 9.4.

NaN_3 was more effective, though much less than KI. The quenching effect of NaN_3 showed the same pH-dependence as KI (cf. fig.2).

3.3. *Aromatic contribution to the lipoxygenase C.D.-spectrum*

Fig.5 shows the near UV circular dichroism spectrum of lipoxygenase-1. This spectrum does not show particular features above 290 nm, while some fine structure is present below this wavelength.

4. Discussion

Little is known about the structure of lipoxygenase and of the ligands and the symmetry of the iron in the protein. Therefore, various possibilities are open to explain the effect of iodide.

The pH dependence of the iodide effects suggests a possible binding of iodide to the metal. Furthermore, the removal of oxygen causes a large increase of the iodide effect, which may indicate that O_2 and I^- compete for the same binding site.

The effects of perturbing agents on the absorbance and the fluorescence of the enzyme are thought to be mainly due to binding of these agents to the iron.

If the fluorescence quenching is assumed to involve a long range energy transfer [5], the distance between the donors and the acceptor can be calculated according to equation (1). For a 50% energy transfer R_0 is about 11 Å at pH 5.3 and 7 Å at pH 9.4. This calculation is reliable for one donor-acceptor pair and for n independent and identical donors with only one acceptor. In the latter case R_0 represents the average distance between each donor and the acceptor [6]. For lipoxygenase-1 this assumption seems to be reasonable since it was impossible to resolve the contributions from the various tryptophan residues to the overall fluorescence. The tryptophan residues in lipoxygenase-1 can be regarded as belonging to Class I according to Burstein et al. [7]: i.e. tryptophans located in a strictly hydrophobic environment. The R_0 values were calculated from equation (1), assuming the

dipole–dipole orientation factor $K^2 = 2/3$ (cf. ref. [8]). The value $K^2 = 2/3$ applies to a fast rotating donor–acceptor pair. This assumption seems to be justified, since the large number of aromatic chromophores [9] shows a relatively low optical activity ($\triangle\epsilon \approx 10$ for the envelope between 256 and 297 nm). Furthermore, the low degree of asymmetry and the absence of fine structure above 290 nm (see fig. 5) suggests, that the contribution from the tryptophans is small. The low asymmetry value may be due either to a non-hindered rotation of the indolyl side chains or to the possibility that differently contributing asymmetric centres cancel each other out. Both possibilities are consistent with the proposed value for K^2, provided that the assumption of equidistance for each tryptophan holds. Anyhow, the average distance between the tryptophan residues and the chromophore produced by iodide binding should be very short. Even a band with a very small absorbance would be able to produce the observed quenching. This would explain the previous finding, that fluorescence quenching by oxygen removal is not accompanied by the formation of a relevant absorption band. However, it is impossible to evaluate the contribution of a dynamic type of quenching by iodide to the overall effect.

On the basis of the present perturbation experiments it is reasonable to propose, that soybean lipoxygenase-1 has a large hydrophobic active site, which contains the tryptophan residues of the protein and iron. The binding of O_2 or anions like I^- and N_3^- invariably results in fluorescence changes due to the close proximity of the tryptophan residues.

Acknowledgements

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