Pyrene Fluorescence Probing of Unsaturated Lipids in *Phytophthora infestans* Zoospores

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Abstract. Pyrene rapidly penetrates into isolated zoospores of phytopathogenic fungus *Phytophthora infestans* localizing predominantly in lipid bodies. An analysis of steady-state monomer and excimer fluorescence spectra, as well as of vibronic structure has suggested a considerable part of the fluorescent probe to be located in a lipid environment. Pyrene partition into hydrophilic phase was observed at its high concentrations. Catalytic hydrogenation of unsaturated lipids in zoospores *in situ* reduced excimer production. The kinetics of changes of pyrene excimerization suggest that hydrogenation affects both the surface and the intrinsic lipids of the zoospores. The usefulness of pyrene as a fluorescent probe for unsaturated lipids in membranes and lipid bodies of intact cells, and the possible role of eicosapolyunsaturated fatty acids in induction of immune response in potato plants are discussed.

Key words: Pyrene excimerization — Unsaturated lipids — *Phytophthora infestans* — Immune response.

Introduction

Pyrene and its derivatives have a wide range of applications in studies of biological structures. A long lifetime in excited state, sensitivity to microenvironment, the ability to form excimers and to serve as energy acceptor, as well as other unique properties make the polycyclic hydrocarbon an useful fluorescent probe for hydrophobic regions of proteins, other macromolecules and biomembranes (Dobretsov 1989; Kalyanasundaram and Thomas 1977; Pownal and Smith 1989; Vekshin 1987). Moreover, current studies tend to extend the approach to intact cells (Dobretsov 1989; Pownal and Smith 1989).

The biotrophic phytopathogenic fungus *P. infestans* is characterized by high

contents of unsaturated lipids containing eicosatetra- and eicosapentaenoic fatty acids. The fatty acids have been believed to directly (or indirectly, via unidentified metabolites) be involved in the elicitation of the defense immune response in potato plants (Golovkin et al. 1989; Merzlyak et al. 1989; Preisig and Kuč 1985). In our recent studies on the role of eicosanoids in the compatibility of partners in the parasite-host plant system we showed that endogenous fatty acids in *P. infestans* zoospores can be modified *in situ* by homogeneous hydrogenation using palladium alizarine complex as the catalyst. This treatment considerably decreased the concentrations of polyunsaturated fatty acids (Merzlyak et al. 1989).

The purpose of the present communication is to evaluate pyrene as a fluorescent probe for unsaturated lipid in intact zoospores of *P. infestans* and to test the effect of the hydrogenation procedure on the physical state of intrinsic and surface lipids of the fungus.

Materials and Methods

Zoospores of *P. infestans* Mont de Bary were isolated as described previously (Golovkin et al. 1989), washed by centrifugation and suspended in distilled water at $1.10^6.ml^{-1}$. Uncorrected fluorescence spectra were recorded using a Jasco FP-550 spectrofluorimeter and 1 cm rectangular quartz cell equipped with magnetic stirrer. The spectra were measured with slits set at 3 and 5 nm for excitation and emission spectra, respectively. Pyrene dissolved in ethanol was slowly added to zoospores as described by Kashulin and Merzlyak (1990). The final ethanol concentration did not exceed 1% (vol/vol). Spectral fluorescence measurements were made at least 5–6 minutes after pyrene addition.

Sonicated dipalmitoyl phosphatidylcholine (PhC, Serva) vesicles were prepared in 10 mmol/l Tris-HCl buffer, pH 7.2 (Kashulin and Merzlyak 1990; 1991).

GLC fatty acid and hydrogenation procedure analysis were performed as described previously (Golovkin et al. 1989; Merzlyak et al. 1989).

Microscopical observations were made with a Ploemopak fluorescent illuminator, filter system A and an Orthoplan Leitz microscope.

Results and Discussion

Microscopic observations indicated that pyrene penetrated into *P. infestans* sporangia and zoospores within several minutes after addition. The microphotograph (Fig. 1) shows that the probe was predominantly located in spherical vesicles (probably oil bodies) and is uniformly distributed within the cytoplasm.

The fluorescence excitation and emission spectra of pyrene added to zoospore suspension are shown in Figs. 2 and 3*A*. Essentially the same spectra were obtained with preparations containing high relative quantities of zoospores or sporangia.

At micromolar concentrations pyrene is known to be soluble in water and produces fluorescence (Fig. 2, see also Dobretsov 1989; Kalyanasundaram and Thomas 1977; Kashulin and Merzlyak 1990; 1991). A strong excimer emission at 470 nm was

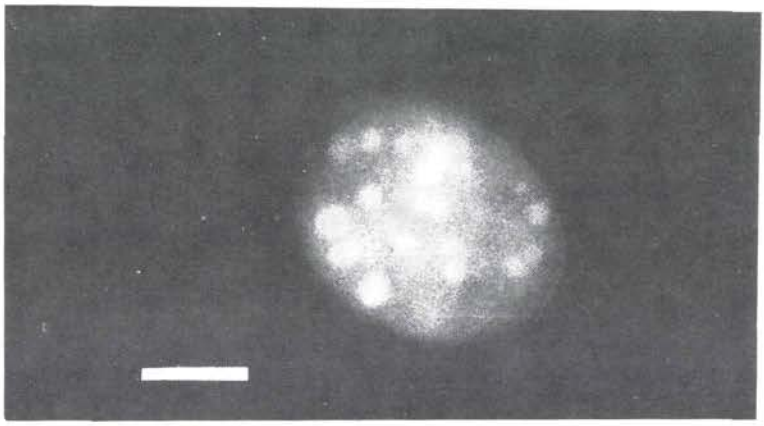

Figure 1. Fluorescence microphotographs of *P. infestans* sporangia 20 minutes after pyrene addition. The bar corresponds to 10 μm.

observed in the presence of zoospores and the emission was enhanced upon increasing pyrene concentration (Fig. 3*B*; no fluorescence in the band was found in water. Previously, we found that the incorporation of pyrene into phospholipid liposomes was accompanied by long wave shift of its excitation fluorescence spectra and the positions of the maxima reflected the partition of the probe between water and lipid phases. It was also shown that the pyrene excitation fluorescence spectrum recorded at λ_{em}=373 nm in PhC liposomes can be represented as a superposition of the corresponding spectra in water and those of excimer excitation spectra in the lipid vesicles (Kashulin and Merzlyak 1991). Probably, similar events occur in zoospores. Excitation maxima recorded both at 470 nm (excimer emission) and at low pyrene concentrations at 393 nm (monomer emission) occurred at approx. 339 nm. Upon increasing pyrene concentration the monomer excitation maximum shifted towards shorter wavelengths, indicating the partition of the probe into water phase at high pyrene/lipid ratios. The excimer excitation maximum that has been attributed to the membrane-bound pyrene population (Kashulin and Merzlyak 1990; 1991) was independent of the probe concentration (Fig. 2, insert).

A comparison of the spectra also shows a strong band near 280 nm in excitation spectrum of pyrene monomers when the probe was incorporated in zoospores (Fig. 2). It is well known that the emission in biomembranes and lipoproteins is due to energy transfer from aromatic amino acids of proteins to pyrene (Dobretsov 1989). The excitation spectrum recorded at λ_{em}=470 nm indicated that the energy transfer reaction does not contribute to pyrene excimerization. Thus, it may be suggested that energy migration from aromatic aminoacids to pyrene incorporated into *P. infestans* zoospores takes place in an environment with a relatively low pyrene concentration and/or mobility as compared to the bulk of the probe.

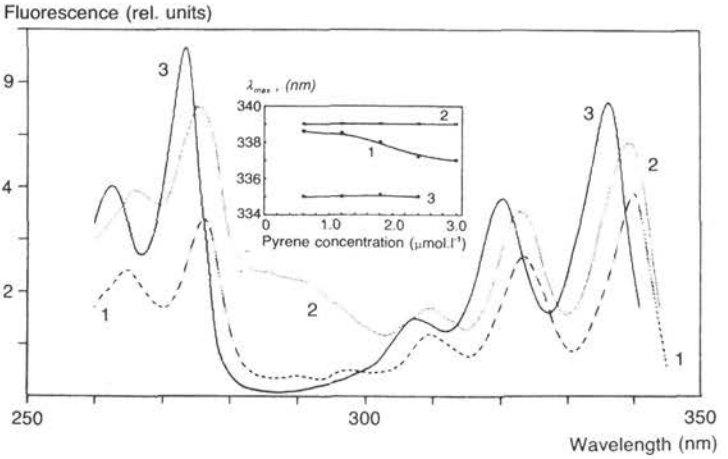

Figure 2. Fluorescence excitation spectra of pyrene in *P. infestans* zoospores (1,2) and in water (3). Spectra 1 and 3 were recorded at λ_{em}=397 nm, and spectrum 2 at λ_{em}=470 nm. Pyrene concentration: 8 mmol.l^{-1}. Insert: The dependence of excitation fluorescence maxima on pyrene concentration. The maxima positions were determined at λ_{em}=373 nm (1,3) or at λ_{em}=470 nm (2).

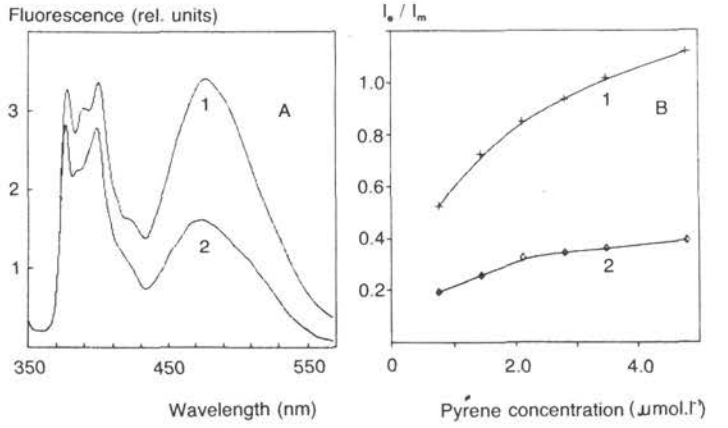

Figure 3. Pyrene excimerization in intact (1) and hydrogenated (2) zoospores of *P. infestans*. *A*: Fluorescence emission spectra recorded at pyrene concentration 10 mmol.l^{-1} and at λ_{ex}=335 nm. *B*: The ratios of excimer (I_e) to monomer (I_m) emission intensities as a function of pyrene concentration.

Table 1. The ratios of pyrene vibronic bands III/I (I_{383}/I_{373}) for different systems

System	This work	Data from Vekshin (1987)
n-hexane	1.49	1.49
Sunflower oil	1.06	–
Rapeseed oil	1.01	–
P. infestans zoospores	1.10	–
Dipalmitoyl PhC liposomes at 46 °C	0.96	–
Dipalmitoyl PhC liposomes at 21 °C	0.86	–
Egg PhC liposomes at 21 °C	–	1.04
Liver mitochondria	–	0.92
BSA	–	0.87
Water	0.61	0.56

The vibronic spectrum of pyrene in singlet state strongly depends on the dipole moment and the dielectric constant of the solvent (Kalyanasundaram and Thomas 1977; Vekshin 1987, also see Tab. 1). In our experiments the ratio of band III (383 nm) to band I (373 nm) in emission spectrum of *P. infestans* zoospores was about 1.10 at low pyrene concentrations. This value is much lower than that in *n*-hexane and higher than those found in egg and dipalmitoyl PhC liposomes at temperatures lower and above phase transition, as well as in liver mitochondria (Tab. 1). Considering intensive fluorescence observed within zoospores in oil bodies (Fig. 1) and the similarities between I_{383}/I_{373} ratios in zoospores and in vegetable oils (Tab. 1), one can suggest that also pyrene emission from neutral nonpolar lipid environment contributes to the total fluorescence of the probe. Thus, the spectral data presented collectively suggest that a considerable part of pyrene is located in hydrophobic regions of *P. infestans* zoospores.

To estimate the advantages of pyrene used as a probe for unsaturated lipids, lipids in zoospores were hydrogenated *in situ* using Pd-alizarine complex as a catalyst. After hydrogenation the relative content of eicosapentaenoic acid decreased from 24.3 to 8.4 mol% (expressed on total fatty acids basis) with simultaneous production of arachidic acid (8.4 mol.%). The contents of stearic, oleic and linoleic acids were 1.2, 11.4, 34.2 and 26.0, 12.5*, 9.5 mol% before and after hydrogenation, respectively (for details see Merzlyak et al. 1989).

Microscopic observations performed as indicated in Fig. 1 did not reveal any differences in pyrene fluorescence distribution between hydrogenated and intact zoospores (not shown).

As already mentioned (Figs. 2 and 3), pyrene added to zoospores displayed

* Including 12-octadecanoic acid produced during hydrogenation

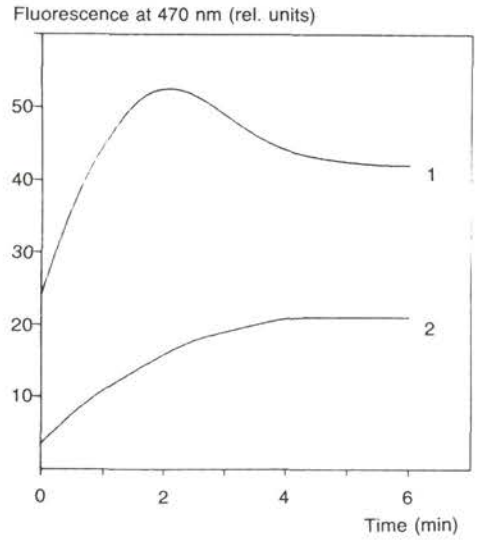

Figure 4. The time course of pyrene excimer emission upon addition to intact (1) or hydrogenated (2) zoospores of *P. infestans*. Pyrene concentration: 10 μmol.l^{-1}.

strong excimerization. The quantum yield of excimer production was considerably higher in intact as compared to the hydrogenated cells both immediately (Fig. 4) and several minutes (Figs. 3*A* and 4) after the fluorescent probe addition. The kinetics of changes of the excimer emission (Fig. 4) shows a considerable enhancement of fluorescence within up to 2 minutes after pyrene addition to intact *P. infestans* cells. The subsequent decrease in pyrene excimerization in this case might be related to its penetration and/or partition into the hydrophobic intracellular compartments (e.g. membranes and oil bodies). In contrast, the kinetics of changes of pyrene excimer emission from zoospores following lipid hydrogenation was monotonic (Fig. 4).

The ratios of steady-state monomer to excimer emission, reflecting both monomer quenching and excimer formation as a result of collisions of the probe in ground and excited states (Dobretsov 1989), increased with the increasing pyrene concentration. Significantly higher I_m/I_{ex} ratios were obtained for intact cells as compared to hydrogenated samples, and the values for intact cells increased more considerably with the increasing pyrene concentration (Fig. 3*B*). These data indicate that as a result of hydrogenation, the bulk of pyrene lipid environment in zoospores turns more rigid.

Immediately after addition of a probe to the cells its fluorescence reflects the binding and interactions with the cell surface (Dobretsov 1989). From this point of view, the lower degree of pyrene excimerization immediately after its addition

to hydrogenated zoospores, as well as the data on the kinetics of pyrene excimer emission (Fig. 4) suggest that hydrogenation affects the unsaturated lipids at the surface of zoospores.

Thus, pyrene fluorescence is a suitable tool for characterizing the physical state of unsaturated lipids in intact cells not only in their membranes but also in oil bodies. This method has routinely been used in our laboratory to control hydrogenation efficiency in *P. infestans* zoospores prior to lipid extraction and fatty acid analysis. Besides, this approach may probably be applied for screening of *P. infestans* races, since the contents of eicosapolyunsaturated fatty acids, exhibiting elicitor activity, were somewhat different within the zoospores of individual isolates of the fungus, and underwent considerable changes during growth of *P. infestans* on artificial nutrient medium (Golovkin et al. 1989).

It is widely accepted that the primary recognition of metabolites responsible for the induction of the plant defense reactions takes place during interactions on the surfaces of pathogen and host-plant cells (Doke et al. 1987). In the context of the hypothesis on the role of eicosapolyunsaturated fatty acids as specific elicitors of phytoimmune response to *P. infestans*, the *in vivo* involvement of the fatty acids in the interactions is of interest. Previously, using hydrogenation with the palladium-alizarine complex, we found that significant conversion of unsaturated (including eicosatetra- and eicosapentaenoic) fatty acids into more saturated species in *P. infestans* zoospores *in situ* did not change the compatibility of the pathogen with potato plants (Merzlyak et al. 1989). The results of the present study indicate that catalytic modification of the fatty acid composition decreases fluidity of both the surface and the intracellular lipids. Thus, it may be suggested that at least absolute levels of unsaturated fatty acids both in total and surface lipids in *P. infestans* zoospores do not directly determine the triggering of the immune response in infected potato plants.

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Final version accepted September 17, 1991