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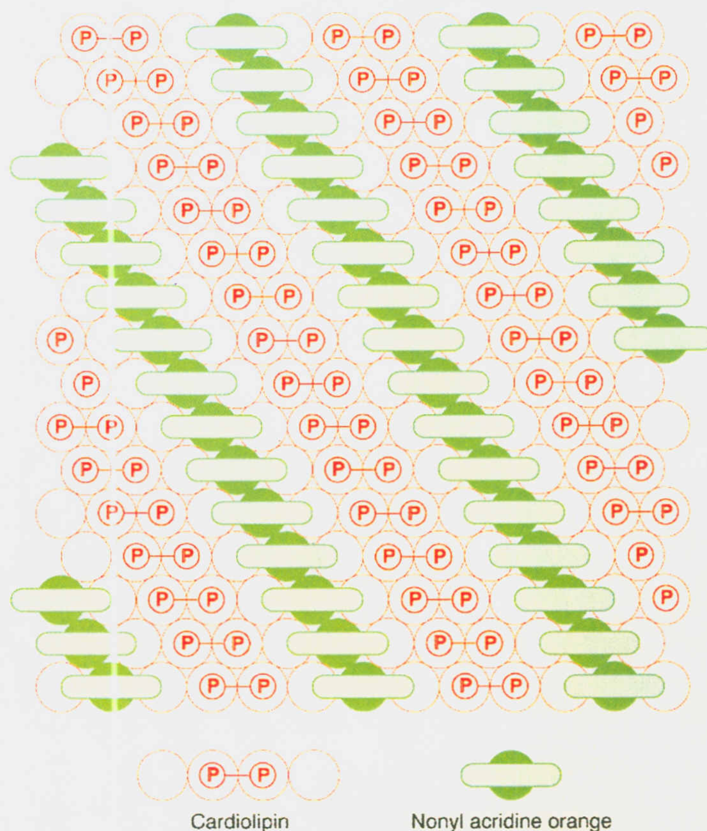

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Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces

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Abstract 10-*N*-Nonyl acridine orange (NAO) has been used at low concentrations as a fluorescent indicator for cardiolipin (CL) in membranes and bilayers. The mechanism of its selective fluorescence in the presence of CL, and not any other phospholipids, is not understood. The dye might recognize CL by its high p*K* (p$K_2 > 8.5$). To investigate that, we established that NAO does not exhibit a p*K* in a pH range between 2.3 and 10.0. A second explanation is that the dye aggregates at hydrophobic domains on bilayers exposed by the CL. We found that a similar spectral shift occurs in the absence of CL in a concentrated solution of the dye in methanol and in the solid state. A model is proposed in which the nonyl group inserts in the bilayer at the hydrophobic surface generated by the presence of four chains on the phospholipid. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: 10-*N*-Nonyl acridine orange; Cardiolipin; Fluorescence emission; Cardiolipin p*K*; Lipid phase separation; *Escherichia coli*

1. Introduction

In the early 1980s 10-*N*-nonyl acridine orange (NAO) was found to penetrate living eukaryote cells and specifically stain mitochondria [1,2]. The staining was soon shown to be located in the inner mitochondrial membrane [3] and was insensitive to the $\Delta\psi$ or to other characteristics of mitochondria-staining dyes [4–7]. As the characteristics of the dye's effect on mitochondria were explored, it became clear that at high concentrations it reduced the level of oxidative phosphorylation and interfered with its enzymes with some specificity. The target appeared to be cardiolipin (CL) [1,8]. Adriamycin, which also inhibits oxidative phosphorylation in mitochondria, has been used under certain conditions by its absorbance ([9] and references therein) to selectively assay for CL. In dilute solutions NAO is a highly specific stain for CL in the mitochondrion and in synthetic phospholipid vesicles although at higher concentrations NAO fluoresces in the presence of phosphatidylserine and phosphatidylinositol in membranes [10]. Nonetheless, because of the dominance of CL in the lipid composition of mitochondria and its access to live mitochondria it has been a useful probe for assessing the CL concentration [11] and indeed the total mitochondrial content in cells [12].

Early studies [10] used NAO's green fluorescence band (525 nm) which was less selective for CL than its red fluorescence which peaks at 640 nm [13]. The properties of the red fluorescence band were rather different from those of the green band. The red band is more intense, more selective for CL than for phosphatidylinositol or phosphatidylserine at low concentrations and displays the unusual property of decreasing its sensitivity to CL at higher concentrations. The fluorescence shifts from 525 nm to 640 nm in the presence of CL-containing bilayers where it overestimates the CL content by 8% in controlled experiments [13].

Recent interest in the use of the dye has stemmed from its ability to identify changes in the distribution of CL in the mitochondrion and in the cell during apoptosis [14–17].

We have examined the binding of NAO to the membranes of yeast mutants [18] and *Escherichia coli* [19]. In *E. coli*, we found that CL occurred in relatively small patches. In an attempt to explore the nature of the association of the dye with CL, we have examined the spectra of NAO in systems that may explain the nature of its specificity for CL.

We have considered two possibilities. One explanation is that, because the pK_2 of CL (> 8.5) is unusually high [20], it is possible that a p*K* of the probe is intermediate between the p*K* of other anionic lipids and that of CL. A second possibility is that, because CL contains four chains and its headgroup appears to be compact [20], it is possible that the NAO ring system fits between the CL molecules in aggregate stacks.

In order to examine the first option we have titrated NAO using its fluorescence to seek a p*K* between pH 2.3 and pH 10.3. To examine the second option we have examined the emission spectra of aggregates of NAO, as it is concentrated in liquid and also in the solid state.

2. Materials and methods

2.1. Chemicals

NAO was obtained from Molecular Probes (Eugene, OR, USA). Buffers were obtained from Fischer Scientific and were diluted with an equal volume of methanol as follows: pH 10.0: 0.05 M K_2CO_3/KOH buffer; pH 7.0: 0.05 M K_3PO_4 buffer; and pH 4.0: 0.05 M potassium biphthalate buffer adjusted to pH 2.3 with HCl. Methanol (spectral grade) was obtained from Mallinkrodt Chemicals.

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Abbreviations: NAO, 10-*N*-nonyl acridine orange; CL, cardiolipin

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2.2. Spectroscopy

Fluorescence measurements of NAO solutions in methanol (1.5 μM–317 mM) were conducted by a model MOS-250 Molecular Kinetics spectrofluorimeter from BioLogic Science Instruments in a glass cell with 2 mm light path. Voltage was 300–500 V; slits were set at 10–20 nm. Excitation (450 nm) was used and the emission spectra were measured between 475 and 700 nm. Fluorescence measurements of NAO in the dry state were conducted by a Fluorolog τ-2 spectrometer (SPEX); detection mode: front face. Excitation, 480 nm; emission measured between 500 and 800 nm. To prepare the dry state NAO samples (3 μl of 150 mM NAO in methanol) were air-dried on a glass slide. The absorption spectra were recorded with a Perkin Elmer UV/Vis spectrophotometer.

3. Results

The emission spectral shift that has been used to examine the binding of NAO to CL-containing membranes is from 525 nm in solution to 640 nm at the membrane surface [13]. In order to understand this spectral shift, we conducted two experiments on the dye itself.

Since the pK_2 of CL is remarkably high compared to all the other phospholipids, we considered that the specificity of the fluorescent dye might be due to a dye pK in the range between that of CL and that of other anionic phospholipids. The results are shown in Fig. 1A. The emission maximum peak was constant at 515 nm using a 1.15 μM solution of NAO dissolved in pH 4.0 (final pH 5.15) and pH 10.0 (final pH 10.3) buffers, each of which displayed the final pH indicated when mixed 50:50 with methanol. The same result was obtained when the sample was dissolved in pH 2.3.

A second explanation for the spectral shift in the emission of NAO when it is bound to CL-containing membranes is that the four chains of the phospholipid provide ample hydrophobic surface on the membrane to permit the insertion of the nonyl tail into the bilayer. This would facilitate direct (π–π) interactions between the aromatic rings. To obtain the spectral shift with increased π–π interactions we obtained emission spectra of a very concentrated solution (Fig. 1B–D) of the dye. It can be seen that the peak shifted from 513 nm at low dye concentration (3 μM) toward 603 nm at the highest concentration that we used (317 mM).

It was possible that, in such a concentrated solution, the sample absorbed photons despite the narrow slit. We therefore measured its absorption spectrum in methanol solutions. It reached saturation at 300 μM. In dilute solutions it displayed a spectral maximum of 488.3 nm. It also exhibited a shoulder at ~460 nm (data not shown).

In view of the high self-absorption in solution, we examined a dry sample devoid of self-absorption. Such a sample would

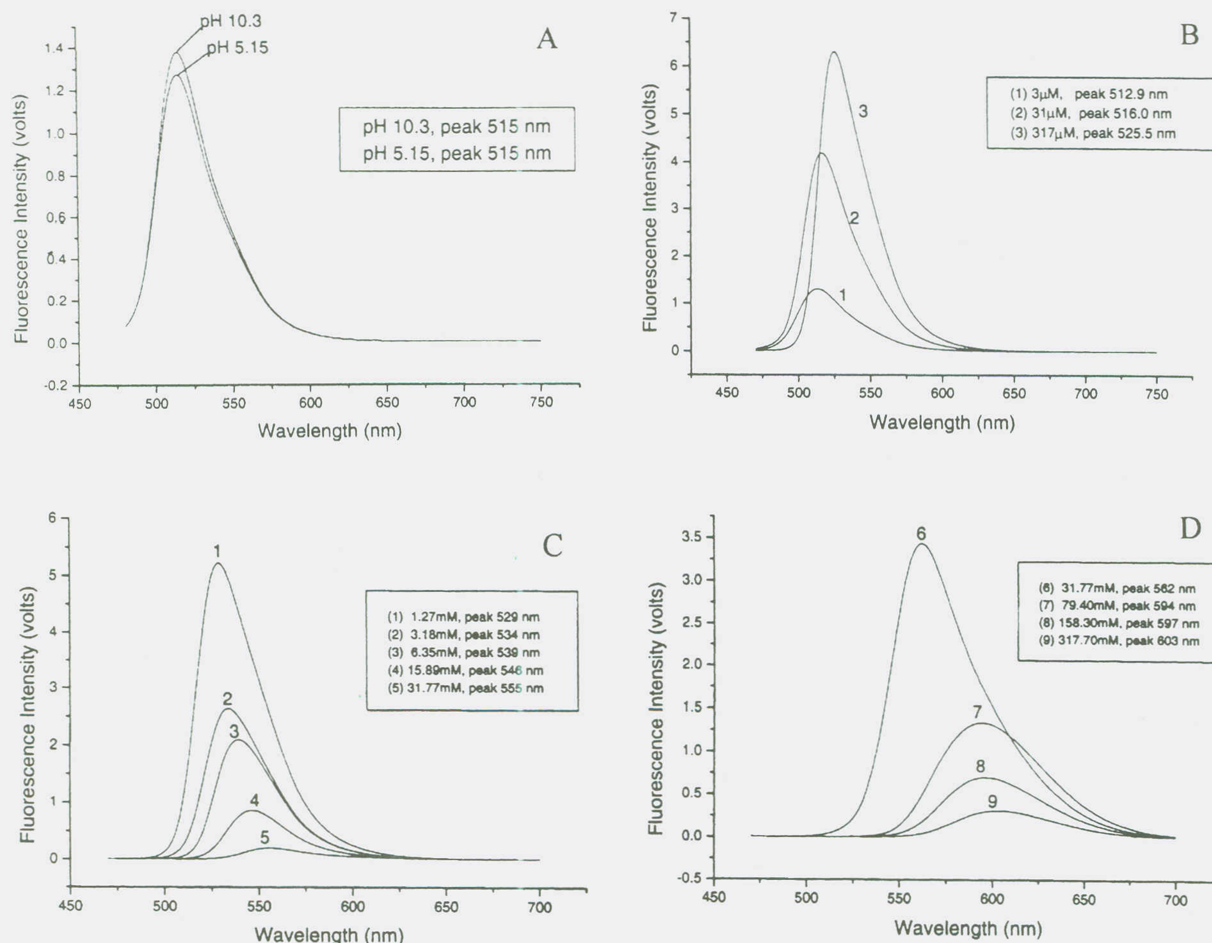

Fig. 1. Fluorescence emission spectra of NAO in methanol at 450 nm excitation. A: 1.5 μM NAO in a 50/50 mixture of methanol and buffer: pH 4.0 (final pH 5.15) and pH 10 (final pH 10.3). B–D: NAO in methanol at the concentrations indicated. The slit width was 10 nm for A–C, and 20 nm for D, for both excitation and emission.

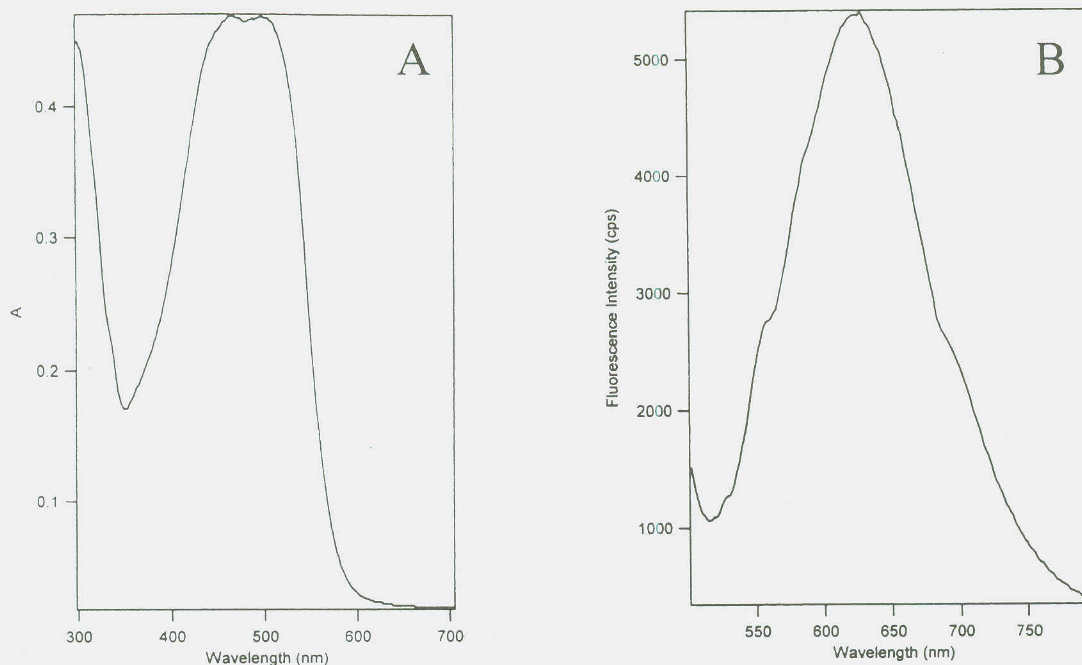

Fig. 2. Spectra for NAO in the dry state. NAO (3 μl of 150 mM) in methanol was dried onto a glass slide in air. A: Absorption spectrum. B: Emission spectrum.

also maximize the π–π interactions of the dye (Fig. 2A). The emission spectrum on a glass slide in front mode (Fig. 2B) of the same sample was obtained using 480 nm for the excitation wavelength. The emission maximum was 624 nm. This is closer than the most concentrated solution emission spectrum to the maximum (640 nm) obtained when the dye is exposed to a CL-containing membrane or vesicle [13].

4. Discussion

4.1. pH titration of NAO

The emission spectra obtained using dilute (1 mM) solutions of NAO in methanol displayed a peak at 515 nm throughout the pH range from pH 2.3 to 10.0 (Fig. 1A). This demonstrates that the dye does not have a pK in this pH range. This experiment was necessary because the pK_2 of CL is greater than 8.0. [20]. This excludes the possibility that the fluorescent spectral shift of NAO in the presence of CL is due to the high pK of the phospholipid.

4.2. Spectral shifts of NAO during concentration

The spectral shift that accompanies the concentration of the dye in methanol (Fig. 1B–D) indicates that NAO displays a fluorescence shift due to its aggregation or 'stacking'. This shift is qualitatively in the direction that NAO displays in the presence of CL whether in vesicles or in mitochondria [13]. However, the interpretation of such measurements may be complicated by self-absorption. The absorption spectrum of the dye in dilute solution exhibits a shoulder near 460 nm. As the concentration in methanol is increased the absorption of this peak goes up dramatically. In the dry state, in the highest concentration (Fig. 2A), the blue-shifted peak (∼460 nm) increases in intensity emerging as a peak of like intensity to the main peak.

The shift in the emission spectrum from 513 nm at 3 μM to 603 nm at 317.7 mM (Fig. 1B–D) shows that there is less and less overlap between the absorption and emission spectra as the dye is concentrated in methanol. The more concentrated the NAO, the higher the wavelength of the emission peak. In dilute solution, its maximum is 513 nm. In the most concentrated solution we used it is 603 nm. In the more concentrated dry state it is 624 nm (Fig. 2B). In bilayers and in mitochondria in the presence of CL, it is 640 nm [13].

Such spectral qualities have also been demonstrated for the binding of acridine orange to nucleic acids [21]. The aggregation of acridine orange to nucleic acids is a result of the spacing to the phosphates on the nucleic acid where the flat dye forms π–π interactions. For this reason the dye displays the same emission spectrum bound to the nucleic acid as it does in concentrated solution. It is here suggested that the aggregated form of NAO in both mitochondria and bilayer surfaces is actually stacking of the monomer to form similar aggregates. In this case the acridine orange plane is bound to the membrane by its nonyl group and not by its cationic charge. Still, it is stacked. This is the case because: first, it displays the aggregated emission spectrum in the absence of the cardiolipin. Second, the nonyl group sits deep in the bilayer with its bromide counterion below the level of the phosphates of the phospholipid. Furthermore, the negative phosphates cannot interact with the cation at its plane or the charge would affect the fluorescence of the probe.

The data suggest instead that NAO fluoresces in the presence of CL-rich domains in bilayers solely by its self-interaction. The dye is clearly binding the bilayer by virtue of the nonyl chain rather than as a cation.

To consider a mechanism for such binding that is unique to CL we present in Fig. 3 an array that takes advantage of the four chains of CL. An implication of our earlier experiments on the pK_2 of CL is that the headgroup of the lipid has a tightly locked, surprisingly small, structure [20]. The two

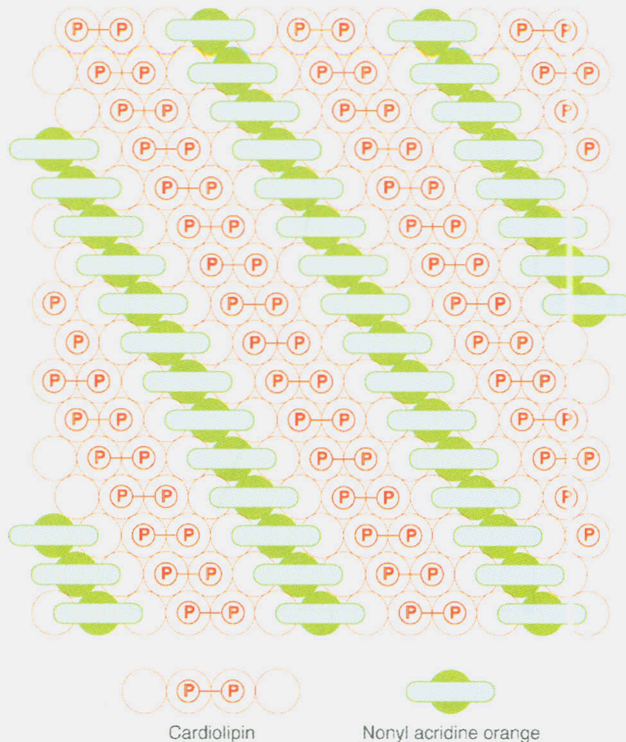

Fig. 3. A proposal for the array of CL in the presence of NAO. This is a top view of the bilayer in which the hexagonal array of large circles represents the fatty acid chains. The small internal circles, containing a P, represent the phosphate groups above the two central chains of the four cardiolipin chains (red). Its bicyclic, H-bonded, and therefore tight headgroup, conformation results from the entrapment of a H^+ from the solution which gives the CL a p$K>8.0$ [20]. This tight array provides room for the NAO (green) to stack in between the CL strings. Our NAO emission data in methanol and in the dry state show that identical stacking occurs with the NAO in the absence of negatively charged lipids. It suggests that the NAO fluoresces at CL surfaces because CL contains four chains and has a small headgroup.

phosphates have trapped a proton and are in a bicyclic array held together by the hydroxyl of the connecting glycerol. This leaves a domain of hydrophobic space (Fig. 3) on the surface of the bilayer between the 'outer' chains of CL giving the probe access to the surface. In this model, the inserted nonyl group provides a stacking arrangement typical of acridine orange. The stacked array in Fig. 3 is stabilized by the π–π interactions between the probe molecules. The pattern is supportive of the tightly locked structure of the headgroup proposed before [20], which provides space between the CL headgroups for the dye molecules. This pattern also explains why the spectra obtained by dry and very concentrated solutions of the probe exhibit the same properties as the spectra obtained from NAO bound to CL domains in fluorescent microscopy. The cardiolipin appears to be organized in clusters on bilayer surfaces. This pattern also explains why NAO does not display red fluorescence with any of the other anionic lipids in bilayers at low concentrations.

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