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Mechanism of acridine orange interaction with phospholipids and proteins in renal microvillus vesicles

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The mechanism of interaction of acridine orange (AO), a fluorescent, weak base, with rabbit kidney brush border membrane vesicles (BBMV) has been studied by absorption, and steady-state and time-resolved fluorescence spectroscopy. Equilibrium binding experiments indicate that AO binds to an apparent single class of sites on BBMV with a dissociation constant of 90 μM and site stoichiometry of 810 nmol/mg protein. The absorption spectra AO indicate that BBMV induces aggregation of AO; experiments with lipid vesicles show that the aggregation requires BBMV membrane proteins. Fluorescence stopped-flow experiments in which 0.15 mg/ml BBMV is mixed with increasing concentrations of AO result in a time course of fluorescence enhancement for [AO] <1.5 μM, and of fluorescence quenching for [AO] >1.5 μM. Similar stopped-flow experiments with phosphatidylcholine lipid vesicles result only in a fluorescence enhancement time cotirse. These results indicate the presence of two parallel pathways for AO binding to BBMV: one for AO binding to BBMV lipid, the other for AO binding to BBMV protein. Nanosecond lifetime measurements and fluorescence titration experiments confirm the presence of two environments for AO in BBMV. Fluorescence stopped-flow experiments indicate that AO responds to the imposition of an outwardly directed proton gradient by a rapid (< 0.5 s) decrease in fluorescence, corresponding to re-equilibration of AO into the acidic intravesicular compartment, followed by an increase in fluorescence, corresponding to proton flux across the membrane. These findings have been incorporated into a stepwise mechanism for AO interaction with BBMV which have direct implications for the use of AO as a pH indicator in biological systems.

1. Introduction

Since its original synthesis by Biehringer in 1896, acridine orange (AO) has been used in a variety of applications, including cellular staining and pH indication in biological systems. AO has been used to measure transmembrane proton fluxes in gastric microsomal vesicles [1], chloroplasts [2], and renal microvillus brush border membrane vesicles (BBMV) [3,4]. Despite the wide use of AO for studying proton transport, a detailed mechanism of the response for interaction of AO with biological membrane systems has not been defined rigorously.

AO is a fluorescent weak base with $pK_a = 10.45$; at physiological pH most AO molecules are protonated. In proton permeability studies, the neutral amine (free base) form of AO is considered to be the only permeable species. In the presence of an outwardly directed proton gradient, AO will accumulate in the acidic intracellular compartment, where at sufficiently high concentrations, AO fluorescence is quenched. As the proton gradient dissipates, intracellular AO returns to the extracellular compartment, resulting in a time-dependent increase in fluorescence.

We have used BBMV isolated from rabbit renal cortex to characterize in detail the AO binding and pH-dependent response mechanism. Equi-

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librium binding experiments indicate that AO binding to BBMV is saturable. Kinetic binding experiments imply that the binding consists of at least two steps. Absorbance measurements allow assignment of binding steps in terms of monomer-multimer equilibria. Fluorescence lifetime measurements confirm the presence of AO in two different environments in BBMV suspensions. The experimental results are incorporated into an explicit mechanism for AO binding to BBMV.

2. Materials and methods

2.1. BBMV preparation

Renal BBMV were prepared by a modification of the method of Booth and Kenny [5]. 2–4 kg New Zealand White rabbits were killed by cervical dislocation and the kidneys immediately excised. The renal artery was flushed with ice-cold saline solution, and the cortex was sliced and then suspended in a buffer containing 200 mM mannitol, 10 mM K^+-Hepes, pH 7.5 (buffer A). Similar results for equilibrium and kinetic experiments were obtained when vesicles were loaded and suspended in buffer A + 150 mM *N*-methylglucamine (NMG)-gluconate buffer. The tissue was homogenized in a Waring blender for 1 min at 4°C, then subjected to three strokes of a Potter-Evehjem homogenizer. $MgSO_4$ was added to a final concentration of 12 mM and stirred at 4°C for 15 min.

Vesicles were isolated by differential centrifugation using a Sorvall RC-5B high-speed refrigerated centrifuge (Du Pont Instruments-Sorvall, Newton, CT) at 4°C. The homogenate was spun at 2700 × *g* for 12 min. The supernatant was drawn off and spun at 27000 × *g* for 24 min. The resulting pellet was resuspended in buffer A with 12 mM $MgSO_4$, and homogenized with three strokes of the Potter-Elvehjem tissue grinder. The low-speed–high-speed homogenization process was repeated twice more. The final pellet was resuspended in buffer A containing no $MgSO_4$. Vesicles were stored in liquid N_2 at approx. 30 mg protein/ml as determined by the method of Lowry et al. [6] and of Bradford [7]. Prior to experiments, BBMV were dispersed through a 27-gauge needle. BBMV were enriched 15-fold in maltase activity and less than 0.3-fold in ouabain-sensitive Mg^{2+}-ATPase activity compared to the crude homogenate.

2.2. Phosphatidylcholine vesicle preparation

Egg yolk phosphatidylcholine (PC) (Sigma, St. Louis, MO, 99%, prepared chromatographically) vesicles were prepared by sonication as described previously [8]. PC suspensions in ethanol were dried in a rotary evaporator and were suspended to a final concentration of 10–15 mM in 100 mM mannitol, 10 mM Na^+-Hepes, 150 mM NaCl (pH 7.5). The suspension was sonicated at 4°C for 1 h under N_2 in an Ultrasonics model C2 sonicator (Heat Systems, Farmingdale, NY). Vesicles were then spun at 50000 × *g* for 30 min to remove excess lipid and particulate debris. Vesicles were stored at 4°C under N_2 and used within 24 h of preparation.

2.3. Equilibrium studies

Fluorescence titrations were performed on an SLM 8000 fluorimeter (SLM Instruments, Urbana, IL) equipped with a double-grating excitation monochromator and a cooled photomultiplier. Aliquots of stock BBMV (AO) solutions were added to solutions of AO (BBMV) in a stirred cuvette thermostatted at 25°C; the maximum correction for dilution was 3%. Fluorescence intensities corrected for inner filter effects (F_{corr}) were obtained from the absorbance of the solution at the fluorescence excitation (A_{ex}) and emission (A_{em}) wavelengths, and the observed fluorescence intensity (F_{obs}) by a correction factor [9]:

$$F_{corr} = F_{obs} 10^{(A_{ex}+A_{em})/2} \quad (1)$$

Equilibrium binding measurements were performed by equilibrating known amounts of AO and BBMV for 30 min at 25°C, followed by centrifugation of the suspension at 50000 × *g* for 20 min. The supernatant was drawn off, and the concentration of unbound AO was determined from the absorbance of the supernatant at 493 nm. The amount of AO bound to the vesicle was

obtained as the difference between the total and free AO concentrations.

Fluorescence lifetime measurements were made on an SLM 4800 phase-modulation lifetime instrument. Lifetime heterogeneity was analyzed using the weighted χ^2 criterion as described by Lakowicz [9]. Some fluorescence lifetimes were also measured on a PRA 3000 pulse lifetime instrument (PRA, London, Ontario). Absorbance measurements were performed on a Hewlett-Packard model 8450A diode array spectrophotometer (Sunnyvale, CA).

2.4. Kinetic studies

Stopped-flow experiments were performed on a Dionex model D-130 stopped-flow apparatus (Sunnyvale, CA) with a dead time of 2 ms. Fluorescence was excited at 493 nm and observed through an Oriel 510 nm long-pass filter (Stamford, CT). The stopped-flow apparatus was interfaced with a Digital MINC 11/23 computer (Maynard, MA) for data acquisition and analysis.

Stopped-flow data, $F(t)$, were fitted to a double-exponential function of the form:

$$F(t) = a_1 e^{(-t/\tau_1)} + a_2 e^{(-t/\tau_2)} + a_3 \quad (2)$$

where a_1 and a_2 represent the amplitudes of the first and second exponentials, τ_1 and τ_2 the corresponding exponential time constants, and a_3 is the baseline offset parameter.

2.5. Materials

All chemicals were obtained from Sigma (St. Louis, MO). AO was purified by recrystallization according to the method of Lamm and Neville [10].

3. Results

3.1. Absorbance measurements

The absorbance spectrum of AO in polar solvents at physiological pH is markedly affected by concentration [11]. Zanker [12] showed that the concentration dependence of AO absorbance spectra at different pH values below the pK_{al} of 10.45 was due primarily to an equilibrium between monomeric (AOH^+) and dimeric ($(AOH)_2^{2+}$) forms of protonated AO:

$$2AOH^+ \Leftrightarrow (AOH)_2^{2+} \quad (3)$$

We have used the spectral properties of the monomer and dimer to observe the species involved in the mechanism of AO binding to BBMV.

Absorbance spectra of AO over the concentration range 18–90 μM at pH 7.5 indicate two dominant absorbing species (fig. 1a). At low concentrations, the monomeric form of AO predominates with an absorbance maximum at 493 nm; as the concentration of AO increases, a dimer peak is observed with an absorbance maximum at 463 nm [10].

The equilibrium dissociation constant for the monomer-dimer equilibrium of AO was determined from the concentration dependence of the intensity of the absorbance peak at 493 nm. The extinction coefficient of the monomer was evaluated from the absorbance of AO in 99% methanol, a solvent in which AO does not aggregate significantly [10]. The concentration of monomer was calculated using Beer's Law and the molar extinction coefficient as determined from AO in methanol. The dimer concentration was calculated as one half of the difference between the monomer concentration and the total AO concentration. The equilibrium dissociation constant of the dimer was calculated as:

$$K_{eq} = ([AOH^+])^2 / \left[(AOH)_2^{2+}\right] \quad (4)$$

The dimerization constant of AO in buffer A determined by this method is 65 μM $\pm$ 8 (S.D., $n = 2$).

In contrast to the protonated species, the absorbance spectrum of AO at pH 11.5 is relatively unaffected by concentration over the range 6–60 μM (fig. 1b). The absorbance spectrum of the neutral amine form of AO is characterized by a single, broad peak with a maximum at 430 nm. These spectra show that the neutral amine form does not aggregate over this concentration range.

Fig. 1c shows the effect of BBMV on the absorbance spectrum of 18 μM AO at pH 7.5; at this concentration, AO is primarily monomeric. Upon addition of BBMV, the intensity of the peak at 463 nm (corresponding to the dimeric, protonated form of AO) increases relative to that of the peak at 493 nm (corresponding to the monomeric protonated form, AOH^+). Addition of 0.1% Triton X-100 to the BBMV-AO solution shifts the spectrum so that the dominant absorbing species is the AOH^+ monomer. Addition of BBMV to solutions containing 1–50 μM AO produced an effect similar to that demonstrated in fig. 1c.

To examine whether dimer formation was induced by BBMV membrane proteins or membrane lipids, the effect of PC lipid vesicles on the absorbance spectrum of 60 μM AO was investigated; at 60 μM, an appreciable concentration of AO dimer is present in solution. Fig. 1d shows that addition of PC vesicles results in an increase of the 493 nm peak, corresponding to a shift in the AO monomer-dimer equilibrium toward monomeric AOH^+. * Addition of lipid vesicles to solutions containing 1–60 μM AO produced similar results.

Because PC vesicles and BBMV membranes respectively shift the equilibrium toward the monomer and dimer, we conclude that the spectral shift observed in fig. 1c upon addition of BBMV to an AO solution results from dimerization of AO induced by BBMV membrane proteins (see below).

3.2. Equilibrium binding

Previous studies of AO binding to BBMV isolated from rabbit renal proximal tubule have shown that the amount of AO taken up by BBMV cannot be accounted for by simple partitioning between intravesicular and extravesicular compartments governed by a pH gradient [13]. To examine in more detail the equilibrium binding of AO to BBMV, we measured the concentrations of bound and unbound AO as a function of total AO and BBMV concentrations by the centrifugation method.

Fig. 2a shows the binding of AO to BBMV over the typical range of concentrations used when measuring proton flux in vesicle systems. The data indicate that over this concentration range there is a constant ratio of bound to unbound AO, indicative of nonsaturable binding of AO to microvillus vesicles. If nonsaturable binding represented partitioning into the entire membrane volume, the partition coefficient (defined as the ratio of the concentration of AO in the membrane to that in the buffer) would be 480, calculated from the normalized slope of fig. 2a.

To investigate saturability, AO binding was also studied over a much larger range of concentrations. As shown in fig. 2b in the form of a normalized Scatchard plot, AO binds to a single, saturable class of sites with a dissociation constant of 90 ± 10 μM and site stoichiometry of 810 ± 30 nmol/mg protein (S.D., $n = 3$). Both the partition coefficient determined from fig. 2a and the large site stoichiometry evaluated via fig. 2b indicate that there is a large reservoir of AO bound to the vesicle membrane.

Table 1 indicates that AO binding to BBMV does not depend on pH over the range pH 5.5–9.5. Over this pH range, the concentration of AOH^+ does not change dramatically, while that concentration of the neutral amine is altered by approx. 4 orders of magnitude. These results indicate that the protonated monomer and protonated dimer are the major components of binding.

3.3. Stopped-flow kinetics

Stopped-flow measurements were used to examine the time course of AO binding to BBMV. For these measurements, AO fluorescence was followed over time subsequent to mixing rapidly AO and BBMV. The fluorescence emission spec-

* A shift toward the peak at 430 nm would be expected if the uncharged form of AO were to partition into the hydrocarbon interior of the PC membrane. A rough estimate of the magnitude of the partition coefficient can be obtained from the ether/water partition coefficient. At pH 7.5, we measured an ether/water partition coefficient for AO of 5.7 ± 0.8. This value is many orders of magnitude too low in order for partitioning of unprotonated AO into PC membranes to account for the spectral shift seen in fig. 1d. Furthermore, if there were appreciable amounts of the neutral amine form of AO present, an absorbance peak at 430 nm would have been observed.

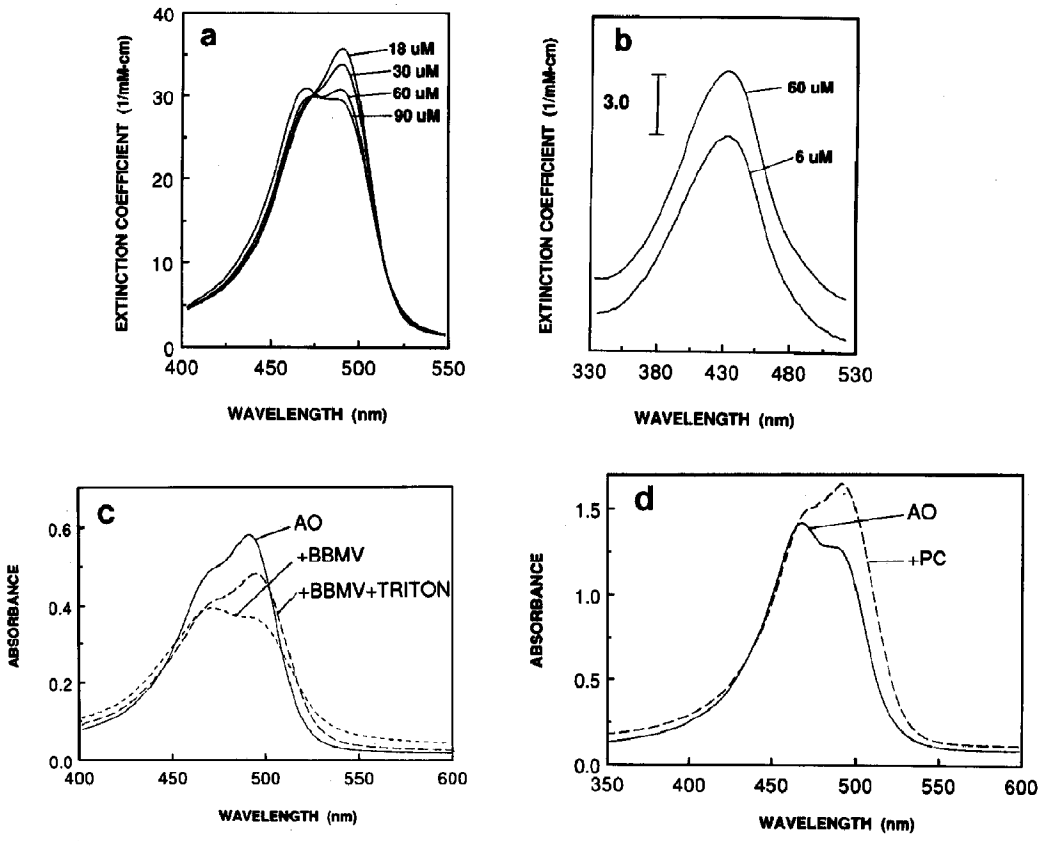

Fig. 1. Absorbance spectra of AO: (a) At varying [AO] in 200 mM mannitol, 10 mM K^+-Hepes (pH 7.5); (b) at [AO] = 6 and 60 μM in 200 mM mannitol, 10 mM K^+-phosphate (pH 11.5). The spectra have been offset along the y-axis for clarity. (c) Effect of BBMV on absorbance spectrum of AO. The solid line is the spectrum of 18 μM AO in 200 mM mannitol, 10 mM K^+-Hepes (pH 7.5). The dashed lines represent the spectra after addition of 0.15 mg protein/ml BBMV to 18 μM AO, and addition of 0.1% Triton X-100 to the AO/BBMV suspension. (d) Effect of PC vesicles on the absorbance spectrum of AO. Absorbance spectrum of 60 μM AO in 100 mM mannitol, 10 mM K^+-Hepes, 150 mM NaCl (pH 7.5) in the presence (dashed line) and absence (solid line) of 1 mM PC vesicles.

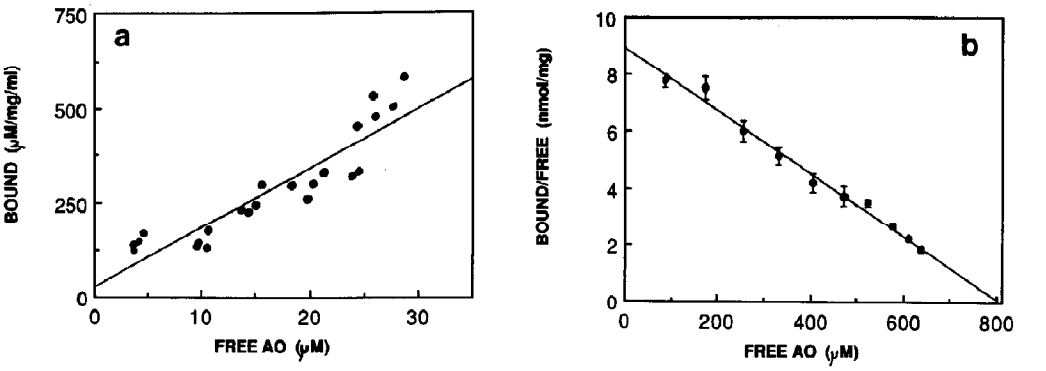

Fig. 2. Equilibrium binding of AO to BBMV. (a) Bound vs. free [AO] over the concentration range 6–36 μM. The slope of a linear regression analysis of the data is 16 ± 0.1 $(mg/ml)^{-1}$. (b) Scatchard plot of AO binding to BBMV. Error bars represent standard deviations from duplicate or triplicate samples. For this set of data, the x-intercept is 785 nmol/mg and the dissociation constant 89 μM.

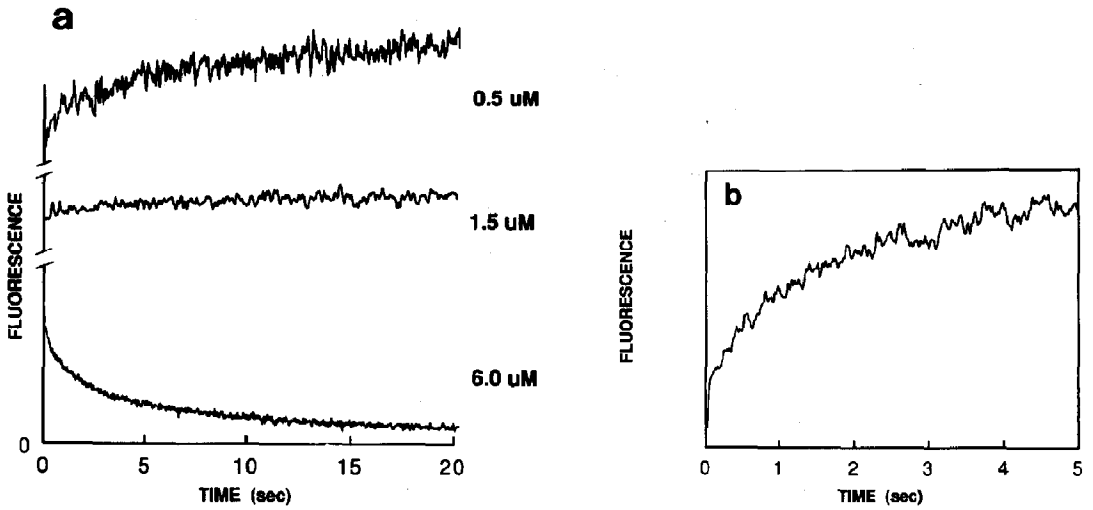

Fig. 3. Time course of AO binding to BBMV and PC vesicles. (a) Top trace: an equal volume of 1.0 μM AO was mixed with 0.30 mg protein/ml BBMV in a stopped-flow apparatus to produce a final [AO] = 0.5 μM and [BBMV] = 0.15 mg protein/ml. AO fluorescence was monitored as described in section 2. Middle trace: 3.0 μM AO was mixed with 0.3 mg protein/ml BBMV. Bottom trace: 12 μM AO was mixed with 0.3 mg protein/ml BBMV. The instrumental gain for the bottom trace was a factor of 10 lower than that of the upper traces. (b) 100 μM AO was mixed with 1 mM PC vesicles. Similar time courses were observed with [AO] over the range 6–150 μM.

trum of AO is maximal at 530 nm, and does not change upon addition of BBMV.

At [AO] < 1.5 μM, a fluorescence enhancement time course was observed when BBMV and AO were mixed (fig. 3a, top). As [AO] increases at constant [BBMV], the amplitude of the time course decreases until it becomes almost flat at [AO] = 1.5 μM (fig. 3a, middle). At [AO] > 1.5 μM, a decreasing fluorescence time course was observed (fig. 3a, bottom). When BBMV are treated with 0.1% Triton X-100, no time course was observed over the concentration range 1–12 μM, indicating that the binding sites responsible for the enhancement and quenching are destroyed by treatment with the detergent, or that the rate-determining steps are made faster than the 2 ms time resolution of the stopped-flow experiment. No fluorescence time course was observed when AO (1–100 μM) was mixed with bovine serum albumin (0.9 mg protein/ml).

Table 1

pH dependence of AO binding to BBMV

AO at specified concentrations was mixed with BBMV at 0.08 mg/ml and equilibrated at 4° C for 15 min, then centrifuged at 40000 × g for 20 min at 4° C. The absorbance at 492 nm of the supernatant was measured and used to determine the value of free [AO]. The percent bound was calculated as the difference between total and free [AO], divided by total [AO]. Errors in percent bound are 5–10%.

$[AO]_{total}$ (μM)	Percent bound		
	pH 5.5	pH 7.5	pH 9.5
6	36	42	35
12	39	42	41
15	42	52	44
21	40	40	44

The change in signal from fluorescence enhancement to fluorescence quenching indicates that the binding mechanism is composed of at least two steps, viz., one leading to enhancement, the other to quenching of AO fluorescence. These two processes may be due to AO binding to the two major components of the vesicle, protein and lipids. To investigate the lipid component of binding, the time course of AO binding to PC vesicles was studied. Over the AO concentration range 1.5–150 μM and at a PC vesicle concentration of 1.0 mM, only fluorescence enhancement was observed (fig. 3b). We therefore assign the fluores-

Table 2

Stopped-flow time constants for AO binding to BBMV

BBMV concentration was 0.15 mg protein/ml. The time constants were obtained from an average of five stopped-flow traces at each AO concentration. Errors are the standard deviation of the fitted time constant for the individual concentration measurements. Errors for the averages are the standard deviations of the sums of the individual measurements.

[AO] (μM)	τ_1 (s)	τ_2 (s)	Amplitude ratio (fast/slow)
0.25	0.168±0.001	8.71 ±0.04	0.32±0.06
0.50	0.168±0.001	4.74 ±0.01	0.43±0.10
0.75	0.136±0.002	2.28 ±0.01	0.24±0.05
3.5	0.052±0.002	0.434±0.01	1.5 ±0.4
4.0	0.050±0.002	0.419±0.001	2.3 ±0.5
4.5	0.043±0.002	0.385±0.001	2.1 ±0.5
6.0	0.051±0.001	0.387±0.001	1.6 ±0.4
12.0	0.056±0.001	0.467±0.001	2.4 ±0.5
24.0	0.054±0.001	0.455±0.001	2.7 ±0.5
Averages			
<1.00	0.157±0.018	–	0.33±0.10
>3.50	0.051±0.004	0.43 ±0.030	2.1 ±0.5

Table 3

Effect of (a) pH and (b) BBMV on fluorescence lifetime measurements

(a) AO fluorescence lifetimes were measured by the phase-modulation method at 30 MHz. All measurements were made against a glycogen reference solution (0 ns lifetime) at 23°C. (b) BBMV were added to AO at 6 μM in buffer at pH 7.5, and 23°C, and the fluorescence lifetimes were measured by the phase and modulation methods. Results are those obtained from a two-component heterogeneity analysis of the lifetime data.

(a) $[AO]_{total}$ (μM)	τ_p (ns)		
	pH 4.5	pH 7.5	pH 11.5
6	1.88±0.03	1.87±0.005	3.72±0.01
60	2.10±0.01	2.25±0.01	3.69±0.01
120	2.44±0.01	2.50±0.01	3.96±0.01

(b) [BBMV] (mg protein/ml)	τ_1	f_1	τ_2	f_2
0	1.88	1	–	–
0.01	1.92	1	–	–
0.075	1.99	0.946	18.2	0.032
0.15	2.11	0.941	17.9	0.059
0.50	2.51	0.898	18.3	0.102

cence enhancement to binding of AO to the lipid component of the BBMV membrane, and the fluorescence quenching to binding of AO to membrane proteins.

Both the fluorescence enhancement and quenching time courses were not described well by a single-exponential function. To characterize the time courses, two time constants were calculated from the time courses by a fit of a double-exponential function. The time constants are given in table 2 as a function of [AO] at constant [BBMV]. AO binding to PC vesicles was characterized by a single-exponential function. The fitted time constant decreased with increasing [AO] (4.23 s at [AO] = 12 μM, 1.60 s at [AO] = 50 μM)

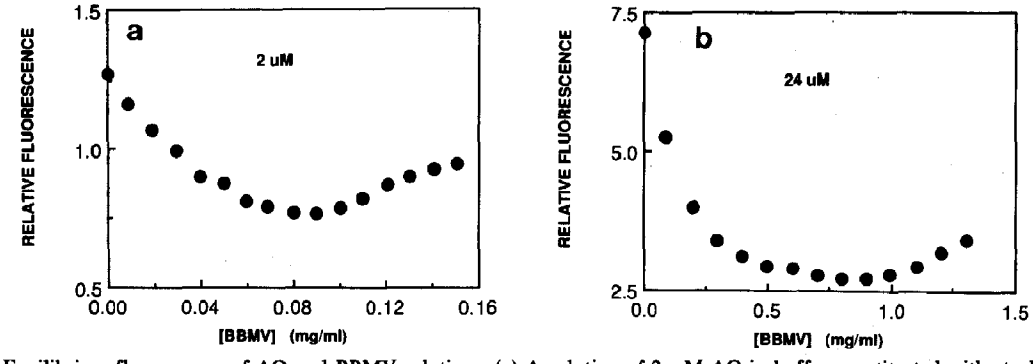

Fig. 4. Equilibrium fluorescence of AO and BBMV solutions. (a) A solution of 2 μM AO in buffer was titrated with stock BBMV. Fluorescence was monitored at 532 nm with excitation at 493 nm. The data were corrected for dilution and inner filter effects as described in section 2. (b) Titration of 24 μM AO with BBMV.

in a manner similar to that of the fluorescence enhancement time courses seen with BBMV.

The relative amplitudes of the two exponential functions used to characterize the fluorescence time course remained relatively constant with increasing [AO] (table 2), even though the overall signal intensity changed by more than an order of magnitude. The abrupt change in relative amplitude from 0.33 for enhancement time courses to 2.1 for those of quenching implies that quenching and enhancement represent two separate processes (see section 4).

The dependence of the stopped-flow quenching time constant on pH was examined. At pH 6.5, the fast and slow exponential time constants for quenching were determined to be 0.12 ± 0.03 and 1.21 ± 0.11 s, respectively, while at pH 8.5, the corresponding values were 0.073 ± 0.010 and 1.50 ± 0.19 s. These values are of the same magnitude as those at pH 7.5 (table 2). At constant total [AO], the time constant should have increased by two orders of magnitude over the range pH 6.5–8.5 if the rate-limiting step were pseudo-first order in unprotonated AO concentration. Therefore, the time constants result from rate-limiting reactions involving the AOH^+ species. A mechanistic interpretation for these results is described below.

3.4. Equilibrium fluorescence measurements

AO fluorescence was measured as a function of [BBMV] at constant [AO]; the results are given in fig. 4. At constant [AO] = 2 μM, addition of BBMV up to 0.09 mg/ml results in fluorescence quenching (fig. 4, top); increasing [BBMV] above this value results in fluorescence enhancement. Similar results are obtained for [AO] = 24 μM (fig. 4, bottom), except that the transition from quenching to enhancement occurs at [BBMV] = 0.90 mg/ml. These data are consistent with the stopped-flow data in which a transition from quenching to enhancement of fluorescence is observed at [AO] = 3 μM when [BBMV] = 0.15 mg/ml, and indicate that at high [BBMV]/[AO], the fluorescence of AO is quenched, while at low [BBMV]/[AO], it is enhanced. From the equilibrium fluorescence measurements, the [BBMV]/[AO] at which the transition from a quenching to enhancement occurs is 0.4–0.5, which is in quantitative agreement with the value of 0.5 determined from stopped-flow measurements, and suggests that there are no significant kinetic processes occurring within the resolution time of the stopped-flow instrument.

3.5. Fluorescence lifetimes

Fluorescence lifetimes of AO were measured in buffer and in BBMV suspensions by the phase-modulation method (table 3a). A single lifetime was obtained for AO in buffer as judged from the equivalence of the phase and modulation lifetimes, and by the lifetimes being independent of modulation frequency. At pH 4.5 and 7.5, where the protonated form of AO predominates, the lifetime is approx. 2 ns, whereas at pH 11.5, where the neutral amine form of AO predominates, the lifetime is approx. 4 ns. These values remained virtually the same over the concentration range 6–120 μM. * Over the same concentration range, AO fluorescence is remarkably quenched. These findings indicate that the dominant mechanism for quenching of AO fluorescence in solution is static quenching in which AO forms a dark complex which does not fluoresce. The dark complex is probably an AO dimer or higher-order aggregate.

When BBMV are added to a solution of AO, the phase and modulation lifetimes are no longer equal. A heterogeneity analysis of the phase shifts and modulation amplitudes revealed a second lifetime of approx. 18 ns (table 3b) in addition to that of 2 ns associated with unbound AO. The

* Lifetimes determined by phase-modulation methods as well as pulse methods can have significant errors due to geometric and/or color effects of the emission photomultiplier tube [9]; these errors will vary as AO concentration varies. To test for the independence of lifetime from AO concentration, lifetimes were also measured at pH 7.5 by the pulse method; these were evaluated as 1.90 ± 0.02, 1.89 ± 0.01 and 2.01 ± 0.03 ns for [AO] = 6, 60 and 120 μM, respectively. In the pulse method used, single photons were detected, and the BBMV concentration was lower than that used in the phase-modulation method. Scattering artifacts, which enhance color effects in photomultiplier tubes, were therefore less in the pulse method.

fraction of this second lifetime component increases with increasing BBMV concentration, indicating that the longer lifetime component is associated with AO binding to BBMV. Since our kinetic results indicate that fluorescence enhancement is observed in binding to PC vesicles and BBMV at low AO concentrations, and since fluorescence enhancement would arise from a lengthened lifetime, we assign the longer lifetime component to AO bound to membrane lipid.

3.6. pH jump experiments

When used as probe of the dissipation of a pH gradient, AO is typically allowed to equilibrate with a membrane or vesicle suspension. The pH of the extracellular solution is then changed to create the pH gradient, and dissipation of the gradient is followed by monitoring changes in AO fluorescence. To define the response time of AO, the time course of AO fluorescence following imposition of a pH gradient was measured in the stopped-flow apparatus.

When BBMV at pH 5.5 are mixed with buffer to give a final extravesicular pH of 7.5 (fig. 5, bottom curve), a biphasic response is observed. The initial rapid drop in fluorescence (time constant = 150 ms) arises from an increase in extracellular pH and subsequent depletion of AO from the fluorescent extracellular compartment and an increase in AO in the nonfluorescent intracellular compartment. The slower increase in fluorescence is due to increasing [AO] in the extracellular compartment as the proton gradient dissipates. In the presence of 50 mM extracellular Na^+ (fig. 5, middle curve), AO fluorescence increases at a more rapid rate, as expected from the enhanced proton permeability due to the Na^+-H^+ antiporter present in BBMV [13].

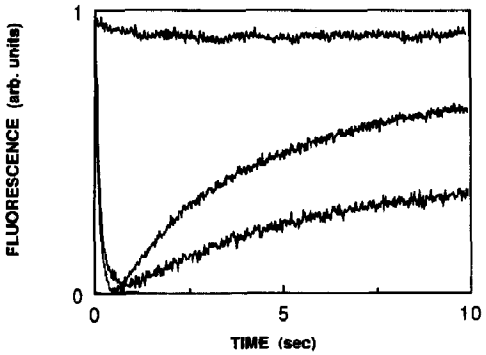

Fig. 5. Response of AO to a proton gradient. BBMV (0.30 mg/ml) were incubated in buffer A at pH 5.5 containing 6 μM AO for 30 min, then mixed in a stopped-flow photometer with an equal amount of buffer at pH 9.5, containing 6 μM AO either with (middle curve) or without (bottom curve) 50 mM NaCl. The time course of AO fluorescence at 523 nm was monitored with excitation at 493 nm. The values of the fitted double-exponential function time constants are 0.130 and 3.51 s (middle curve) and 0.180 and 5.11 s (bottom curve). The top curve is the observed time course when a solution of pH 5.5 buffer with 6 μM AO and 0.30 mg/ml BBMV is mixed with a solution of pH 5.5 buffer and 6 μM AO.

In the stopped-flow experiment, the two solutions to be mixed each had the same total concentration of AO. However, BBMV were added to the pH 5.5 solution so that the extracellular [AO] was reduced due to binding of AO to the BBMV membrane. Therefore, immediately after mixing the buffer and BBMV solutions in the stopped-flow apparatus, AO was no longer in equilibrium with respect to binding to BBMV. Any binding of AO to BBMV could thus lead to a time course of fluorescence separate from that observed from re-equilibration of AO due to the imposition and subsequent dissipation of the initial pH gradient. In order to test for this possibility, an identical stopped-flow experiment was performed in the absence of a pH gradient. The results (fig. 5, top curve) indicate that some binding does occur but at [AO] = 6 μM the additional time course is not of sufficient magnitude to affect appreciably that arising from proton transport.

4. Discussion

We have investigated the mechanism of interaction of AO with BBMV. The interaction mechanism consists of an equilibrium among the intracellular and extracellular neutral amine form, protonated monomer and dimer, and probe bound to BBMV lipids and proteins.

Analysis of the concentration-dependent absorption spectra of AO in buffer solution gave a monomer-dimer equilibrium constant of 65 μM, which agrees well with the values of 41 μM [12],

95 μM [10], and 66 μM [14] reported previously. Therefore, at the concentrations used for most studies of proton transport with outwardly directed proton gradients (5–10 μM), AO in the extracellular solution exists primarily as a monomer. Furthermore, given the pK_{al} of 10.45, virtually all AO monomers will be protonated at physiological pH.

Several experimental findings suggest that AO interacts with membrane lipids. The absorbance spectra of AO show a shift in intensity toward the 493 nm maximum corresponding to AOH^+ monomer when PC vesicles are present (fig. 1d). Fluorescence enhancement is seen in kinetic binding studies with PC vesicles (fig. 3c). With BBMV at low [AO], fluorescence enhancement is also observed in measurements of both kinetic binding (fig. 3) and equilibrium binding (fig. 4b). The time constants characterizing the fluorescence enhancement seen with PC vesicles and BBMV both show a similar dependence on [AO]. Fluorescence lifetime measurements reveal a second, longer lifetime component whose fraction increases with BBMV concentration (table 3b), corresponding to AO in an environment of higher quantum yield. The similarity between experiments performed with BBMV and PC vesicles at low [AO] suggest that AO interacts with BBMV lipids.

Experiments carried out at high [AO] reveal differences between AO binding to PC vesicles and BBMV. When BBMV are mixed with 18 μM AO (fig. 1c), an increase in the absorbance peak corresponding to the AO dimer (463 nm) and a decrease in that corresponding to the AO monomer (493 nm) are observed. Similar experiments with 60 μM AO and PC vesicles give the opposite result: an increase in the monomer absorbance peak and a decrease in the dimer peak. Stopped-flow experiments with BBMV and [AO] > 3 μM resulted in quenching time courses, while PC vesicles gave only enhancement time courses. The transition between AO fluorescence enhancement and quenching was also observed when BBMV were titrated into an AO solution: AO fluorescence initially decreases, then increases as more BBMV are added (fig. 4).

At low concentrations, AO binding to BBMV and PC vesicles is similar; at high concentrations, AO binding to BBMV leads to a shift in the monomer-dimer equilibrium toward the dimer and to fluorescence quenching. Since the major difference between PC vesicles and BBMV is that the latter contains membrane protein, it is plausible to assume that BBMV membrane proteins induce aggregation of AO, leading to fluorescence quenching. At low [AO]/[BBMV], lipid sites are bound leading to fluorescence enhancement; at high [AO]/[BBMV], protein sites become available leading to fluorescence quenching.

The observation of a null time course rather than a biphasic type in stopped-flow experiments in which AO fluorescence changes from enhancement to quenching (fig. 3) implies that the binding steps leading to enhancement and quenching cannot occur sequentially. A likely alternative is that the reaction steps bifurcate so that the binding to lipid and to protein can occur independently.

Taken together, our results support a mechanism for AO interaction with BBMV which must incorporate the following considerations. (1) At AO concentrations used for protein transport measurements, the dominant species in solution is monomeric AO. (2) At pH values used for proton transport measurements, most AO molecules are protonated and charged (AOH^+). (3) AO, AOH^+ and $(AOH)_2^{2+}$ all bind to BBMV. (4) The dominant transported species across the hydrocarbon interior of the membrane is uncharged AO. (5) Monomeric AOH^+ binds to membrane lipids, leading to fluorescence enhancement. (6) Dimeric $(AOH)_2^{2+}$ binds to membrane proteins, leading to fluorescence quenching. (7) Binding steps leading to enhancement and to quenching cannot occur sequentially.

We have incorporated these requirements into a proposed mechanism for AO binding to BBMV (fig. 6). While the reaction mechanism is not unique, it does represent a plausible, minimal mechanism which explains the experimental observations. All reactions in the extracellular and intracellular solutions are fast; protonation of the free base and dimerization of the protonated monomer occur within a time of the order of microseconds or less [14] and therefore take place within the dead time of the stopped-flow measurement.

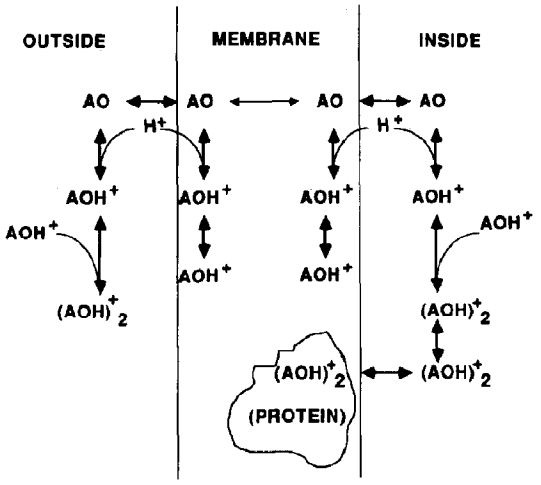

Fig. 6. Proposed model for AO binding to BBMV.

In the absence of a pH gradient and at low [AO], the only species present are AO and AOH^+; given consideration 5 above, these species must interact with membrane lipid. Consideration 2 implies that the dominant species in the lipid is AOH^+. The observation of two time constants for binding (table 2) implies that there is not a single slow unimolecular or pseudo-unimolecular step characterizing AO binding to lipid. Since consideration 7 implies that slow enhancement steps cannot precede quenching steps, a slow 'dead-end' step is added for AOH^+ bound to the outer and inner surfaces of the membrane; these steps lead to fluorescence enhancement. These slow steps may represent a deeper intercalation of AOH^+ within the lipid portion of BBMV, leading to a more hydrophobic environment and reduced motional freedom that give rise to fluorescence enhancement. Consideration 7 also dictates that the rate of transport of AO from one side of the membrane to the other cannot be slow.

Consideration 6 implies that as the AO concentration increases, protein binding sites become available, and fluorescence quenching is observed. Since two time constants are observed for quenching (table 2), quenching does not occur in a single slow unimolecular or pseudo-unimolecular step. The protein binding sites are accessible from the inside of the vesicle, consistent with the observations of Warnock et al. [13].

Numerical simulations of the mechanism in fig. 6 indicate that within the signal-to-noise limitations of the fluorescence signal, an increase in AO concentration will smoothly change the observed kinetic stopped-flow traces from an enhancement time course, through a null time course, to a quenching time course.

How does AO respond to a pertubation of pH in the vesicle system? The transported species of AO is the unprotonated, neutral amine form; the weak base character of the neutral amine form of AO will allow it to concentrate preferentially in an acidic intracellular compartment. The larger number of protein binding sites accessible from the intravesicular compartment will allow much more AO to accumulate intravesicularly than predicted from the proton gradient, effectively increasing the size of the intravesicular compartment. As protons are transported from inside to outside the vesicle, thereby changing the relative concentrations of AO and AOH^+ on the two sides of the membrane, the equilibrium system in fig. 6 will shift to the left: nonfluorescent $(AOH)_2^{2+}$ will unbind from membrane proteins, dissociate and deprotonate to AO. AO will be transported across the membrane to the extracellular solution, resulting in a net increase in fluorescent AO of the suspension.

The results presented here have several direct implications for the use of AO in measuring proton transport in vesicle and cell membrane systems. AO is commonly used at a concentration of about 6 μM to observe proton flux in BBMV vesicle systems [13,15,16]. Our data indicate that saturation of the inner binding sites occurs at approx. 130 μM AO. Consequently, a perturbation of 1.5–2 pH units around physiological pH will not saturate intravesicular protein binding sites at [AO] = 6 μM. At significantly higher [AO], however, the relation between bound and free sites is no longer linear (cf. fig. 2a and b). If experiments are performed at higher [AO], the effective size of the intravesicular compartment changes as AO flux across the membrane occurs. For other vesicle systems, binding experiments similar to those in fig. 2a should be carried out in order to ascertain that [AO] remains on the linear part of the binding curve throughout the time course of

proton flux. This precaution will simplify the kinetic analysis.

As protons flow across the membrane in response to a pH perturbation, AO will re-equilibrate. Dissipation of an outwardly directed proton gradient will result in accumulation of much more AO in the extravesicular space than predicted from the intravesicular volume. Because AO bound intravesicularly to protein provides an additional supply of quenched fluorophore to be transported out and converted into the fluorescent form, the apparent intravesicular space is much larger than that calculated from the vesicle dimensions (approx. 0.1 μm^3). pH jump experiments similar to those performed with BBMV were unsuccessful when performed with pure lipid vesicles, since the total amount of intravesicular, quenched AO in pure lipid vesicles is much less than in BBMV. Therefore, in order for AO to be a sensitive probe, the membrane system must have intravesicular protein sites which can bind AO, lead to fluorescence quenching, and increase the effective size of the intravesicular compartment. A corollary is that [AO] cannot be arbitrarily low, but must be high enough to populate appreciably the protein binding sites without saturating these sites.

Our data indicate that the response time of AO as an indicator of proton flux is limited by the observed time course of binding, approx. 0.5 s in BBMV when protein sites provide the intravesicular pool of AO. If [AO] is low so that the intravesicular pool comprises lipid sites, then the response time is longer, of the order of many seconds (table 2). For membrane systems other than BBMV, separate kinetic experments similar to those described here are required to show that the signal due to proton flux is not limited by the response time of AO.

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References

1 H.C. Lee and J.G. Forte, Biochim. Biophys. Acta 508 (1978) 339.
2 S. Schuldiner, H. Rottenberg and M. Avron, Eur. J. Biochem. 25 (1972) 64.
3 W.W. Reenstra, D.G. Warnock, V.J. Yee and J.G. Forte, J. Biol. Chem. 256 (1981) 11663.
4 H.E. Ives, V.J. Yee and D.G. Warnock, J. Biol. Chem. 25 (1983) 9710.
5 A.G. Booth and A.J. Kenny, Biochem. J. 142 (1974) 575.
6 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
7 M.M. Bradford, Anal. Biochem. 72 (1976) 248.
8 C. Huang and T.E. Thompson, Methods Enzymol. 32 (1974) 485.
9 J.R. Lakowicz, Principles of fluorescence spectroscopy (Plenum, New York, 1983).
10 M.E. Lamm and D.M. Neville, J. Phys. Chem. 69 (1965) 3872.
11 S.E. Sheppard, Proc. Roy. Soc. A82 (1909) 256.
12 V. Zanker, Z. Phys. Chem. 199 (1952) 225.
13 D.G. Warnock, W.W. Reenstra and V.J. Yee, Am. J. Physiol. 242 (1982) F733.
14 G.G. Hammes and C.D. Hubbard, J. Phys. Chem. 70 (1966) 1615.
15 I. Sabolic and G. Burckhardt, Biochim. Biophys. Acta 731 (1983) 354.
16 E.S. Foster, P.K. Dudeja and T.A. Brasitus, Am. J. Physiol. 250 (1986) G781.