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SYNTHESIS OF 5-NITRO-2-[N-3-(4-AZIDOPHENYL)-PROPYLAMINO]-BENZOIC ACID: PHOTOAFFINITY LABELING OF HUMAN RED BLOOD CELL GHOSTS WITH A 5-NITRO-2-(3-PHENYLPROPYLAMINO)-BENZOIC ACID ANALOG

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SUMMARY: A photoaffinity analog of the potent epithelial chloride channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid has been synthesized and characterized. In the dark, this reagent, 5-nitro-2-[N-3-(4-azidophenyl)-propylamino]-benzoic acid, and the parent compound reversibly inhibited chloride efflux in human red blood cell ghosts. Irradiation of ghost membranes with 350 μM arylazide analog reduced the rate of chloride efflux to 33% of the control value. The photoinactivation process was not reversed by exhaustive washing of ghost membranes. Covalent incorporation of the photoaffinity reagent was supported by difference ultraviolet spectroscopy, which indicated the attachment of the substituted 2-amino-5-nitrobenzoic acid chromophore to ghost membranes. The novel photolabeling agent described here should be a useful structural probe for chloride channels in erythrocyte membranes and epithelial cells.

INTRODUCTION: Epithelial cells, such as those found in human airway, can secrete and reabsorb fluid. Water movement is intimately coupled to Na^+ and Cl^- transport in these processes (1). In CF, a common lethal inherited disease among Caucasian populations, airway and other epithelia are anion impermeable (2) and Cl^- channel regulation by cAMP is defective (3). A clearer understanding of the structure, function and regulation of Cl^- channels in epithelia is foremost to a complete determination of the biochemical manifestation of CF.

Studies on Cl^- transport proteins using several groups of synthetic inhibitors suggest that many of these proteins belong to one family (4). NPPB (Figure 1) is the most potent member of an important class of reversible blockers of a wide variety of epithelial Cl^- channels (5,6). Anion exchange in human erythrocytes also is blocked by NPPB (7), and it is likely that the Cl^-/HCO_3^- exchanger (band 3 protein) is structurally similar to the family of epithelial anion transport proteins. Our aim is to develop photoaffinity labeling agents structurally similar to NPPB for eventual use as probes for Cl^- channel structure. In this communication, we report the synthesis of AzNPPB (Figure 1), an aryl azide analog of NPPB. Initial evaluation of this synthetic photoprobe using human erythrocyte ghosts indicates that it is a very promising reagent for the study of Cl^- channels.

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Abbreviations: Ac, acetyl; AzNPPB, 5-Nitro-2-[N-3-(4-azidophenyl)-propylamino]-benzoic acid; CF, cystic fibrosis; DNDS, 4,4'-dinitro-2,2'-stilbene disulfonate; DMSO, dimethyl sulfoxide; NPPB, 5-Nitro-2-(3-phenylpropylamino)-benzoic acid; and SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS: *Materials.* 2-Fluoro-5-nitrobenzoic acid (8) and NPPB (5) were prepared by literature procedures. 3-(4-Nitrophenyl)-1-phthalimidopropane was synthesized according to Parker *et al.* (9) from 3-(4-nitrophenyl)-1-bromopropane and potassium phthalimide (Aldrich) by modifying the heating conditions to room temperature for 2 h followed by 1 h at 50 °C. The bromo compound was prepared by the method of Davis *et al.* (10) from 3-phenyl-1-bromopropane (Aldrich) except that the nitration mixture was maintained at 5-10 °C followed by stirring at room temperature for 2 h. DNDS was from Pfaltz & Bauer and $H^{36}Cl$ (13.0 mCi/g Cl) from ICN Biomedicals.

General methods. Ultraviolet-visible spectra were taken with a Perkin-Elmer Model Lambda 4A or 3B spectrometer interfaced to an Analog Connection Workstation (Strawberry Tree Computers, Inc.) and a Macintosh SE computer. Infrared spectra were obtained using a Perkin-Elmer Model 1600 FTIR. ^{1}H- and ^{13}C-NMR spectra were measured at 250.13 and 62.90 MHz, respectively with a Bruker AC-250 instrument, and values are reported relative to TMS. The resonance frequency for the ^{35}Cl-NMR studies is 24.51 MHz. Elemental analyses and high resolution mass spectra using a VG 70/250S were provided by Pfizer Central Research. Chloride-36 radioactivity was measured on a LKB-Wallac Model 1214 liquid scintillation counter. Melting points (uncorrected) were obtained with a Mel-Temp apparatus.

Erythrocyte ghost preparation. Freshly outdated human blood (packed red cells) was obtained from the Connecticut Chapter of the American Red Cross. Resealed erythrocyte ghosts and leaky ghost membranes were prepared according to Steck and Kant (11). Total ghost protein was determined by a modification (12) of the Lowry method (13).

Chloride flux measurements. Chloride-36 efflux measurements with resealed erythrocyte ghosts in 20 mM Tris, pH 7.2 containing 150 mM NaCl and 10 mM KCl (efflux buffer) were made according to the method of Gunn and Fröhlich (14). Ghost suspensions (1-2 mg protein) were incubated for 30 min in 0.5 mL of efflux buffer containing $^{36}Cl^-$ (final specific activity 0.5-2 μCi/mL), centrifuged at 16000 X *g*, and the resulting packed ghosts (0.1-0.2 mL) were injected into 30 mL of efflux buffer with stirring at 0-1 °C. Samples (~ 0.7 mL) of ghost-free medium were withdrawn at various time intervals up to 30 s using Swinnex syringe filters. Radioactivity in 0.5 mL aliquots was determined by liquid scintillation counting. The appearance of $^{36}Cl^-$ in the ghost-free medium with time was used to determine the rate constant for chloride self-exchange (14). Rate constants for control resealed ghost preparations were 0.024-0.030 s^{-1}. In studies to evaluate reversible inhibition, AzNPPB and NPPB in DMSO stock solutions were added to ghost suspensions (final DMSO concentration was 1%).

Irradiation procedure. To suspensions of resealed ghosts in efflux buffer (0.5-1.0 mg protein/mL) were added DMSO solutions of AzNPPB (with or without NPPB), or prephotolyzed solutions of AzNPPB, or solvent alone (1%) in quartz cuvettes at 5 °C. Nitrogen was bubbled into the suspensions and the cuvettes were placed 4 cm from a RPR-2537 (Southern New England UV) "254 nm" ultraviolet source. The mixtures were stirred gently while being irradiated for 3-5 min. Irradiated samples were washed twice with 10 volumes each of efflux buffer, efflux buffer containing 0.5% bovine serum albumin, and efflux buffer.

^{35}Cl-NMR spectroscopy. Measurements of apparent K_D values for NPPB and AzNPPB using leaky membranes (1.5-3.5 mg protein/mL) were performed as described by Falke *et al.* (15) except that the NMR buffer was 20 mM sodium phosphate, pH 8.0 containing 62.5 mM NaCl. Stock solutions of inhibitors (17 μM) in DMSO were added in 1 μL aliquots (up to 8 μL/mL) to suspensions of leaky membranes in NMR tubes. A correction was made for solvent induced linebroadening (0.3 Hz/μL). For ^{35}Cl-NMR line width measurements, the spectral width was 2000 Hz containing 1 K data points, centered on the solution chloride peak. Using 5 mm tubes, 512 pulses were accumulated at 21 °C using a 90° pulse (12.4 μs) and a 0.1 s repetition delay. Spectra were acquired without sample spinning or deuterium lock. A linebroadening of 10.0 Hz was applied with zero filling to 4 K. The central 260 Hz of the spectra were displayed and the line width at half height was calculated with the Bruker curve-fitting routine for Lorentzian peaks.

3-(4-Aminophenyl)-1-phthalimidopropane **(III).** To a solution of 3-(4-nitrophenyl)-1-phthalimidopropane (1.6 g, 5.16 mmol) in EtOAc (100 mL) was added 50 mg of 5% Pd/C catalyst. The mixture was transferred to a Parr Hydrogenator and vigorously shaken for 3 h under a H_2 atmosphere (3.4 atm). The mixture was filtered through a Celite pad, washed with EtOAc, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield 1.4 g (97%) of yellow solid. The crude product was recrystallized from ethanol yielding 1.2 g of yellow needles (4.0 mmol, 78%) with mp 113-114 °C. IR (KBr, cm^{-1}) 3436, 3359, 1773, 1703, 836 and 726; ^{1}H-NMR ($CDCl_3$) δ 7.81 (AA'XX', 2H, ArH *ortho* to C=O), 7.71 (AA'XX', 2H, ArH *meta* to C=O), 6.98 (AA'XX', 2H, ArH *meta* to NH_2), 6.60 (AA'XX', 2H, ArH *ortho* to NH_2), 3.71 (t, 2H, J = 7.2 Hz), 3.40 (s, 2H, NH_2), 2.57 (t, 2H, J = 7.8 Hz), and 1.96 (2t, 2H,

J = 7.2, 7.8 Hz); ^{13}C-NMR ($CDCl_3$) δ 30.2, 32.3, 37.8, 115.3, 123.2, 129.2, 131.1, 132.2, 133.9, 144.3, and 168.5. HRMS (EI) calcd. for $C_{17}H_{16}N_2O_2$ 280.1213; found 280.1233.

3-(4-Azidophenyl)-1-aminopropane - Phthalazin-1,4-dione complex (**IV**). To a stirred solution of aryl amine **III** (585 mg, 2.1 mmol) in acetone (40 mL) was added dropwise over 10 min 2N HCl (20 mL). The mixture was cooled to 0 °C and a solution of sodium nitrite (196 mg, 2.8 mmol) in H_2O (8 mL) was added dropwise over 10 min. Stirring was continued at 0-4 °C for an additional 30 min. From this point, all procedures were done in the dark. The mixture was poured into a cold aqueous solution (40 mL) containing sodium azide (1.82 g, 28.0 mmol); Et_2O (100 mL) was added, and the mixture was stirred for 30 min without cooling. The Et_2O layer was set aside and the aqueous layer was extracted with Et_2O (2 X 20 mL). The organic layers were combined, washed with H_2O (2 X 20 mL), and dried over anhydrous Na_2SO_4. The solvent was evaporated *in vacuo* to yield a yellow solid: tlc (silica gel, $CHCl_3$:EtOAc, 2:1, v/v) R_f = 0.88; IR (KBr, cm^{-1}) 2110, 1770, 1713, 828 and 722. The protected aryl azide was dissolved in anhydrous CH_3OH (11 mL) and anhydrous hydrazine (0.368 mL, 11.5 mmol) was added with stirring under N_2 at room temperature. After 1 h, the mixture was rotary evaporated and dried overnight *in vacuo* . The product was obtained as a pale yellow solid (560 mg, 1.66 mmol, 79%) with mp 158-160 °C (dec.) and was stored at -20 °C . ^{1}H-NMR (DMSO-d_6) δ 8.06 (AA'XX', 2H, Ar<u>H</u> *ortho* to C=O), 7.79 (AA'XX', 2H, Ar<u>H</u> *meta* to C=O), 7.25 (AA'XX', 2H, Ar<u>H</u> *meta* to N_3), 7.02 (AA'XX', 2H, Ar<u>H</u> *ortho* to N_3), 5.13 (br s, N<u>H</u>$_2$, N<u>H</u>-N<u>H</u>), 2.62 (2 overlapping t, 4H), and 1.73 (2t, 2H); ^{13}C-NMR (DMSO-d_6) with DEPT δ 31.6 (t), 32.8(t), 39.8 (t), 118.9 (d), 125.2 (d), 128.3 (s), 129.8 (d), 131.7 (d), 138.5 (s), 156.0 (s), and 169.0 (s); tlc (silica gel, $CHCl_3$:CH_3OH, 8:2, v/v) R_f = 0.75. HRMS (FAB) MH^+ calcd. for $C_{17}H_{19}N_6O_2$ 339.1572; found 339.1570.

5-Nitro-2-[N-3-(4-azidophenyl)-propylamino]-benzoic Acid (**I**). All procedures were done in the dark. Aryl azide **IV** (338 mg, 1 mmol), 2-fluoro-5-nitrobenzoic acid (167 mg, 0.9 mmol), and N,N-diisopropylethylamine (0.27 mL, 2.0 mmol) were dissolved in N,N-dimethylacetamide (3 mL) with stirring under N_2. The mixture was heated at 100 °C for 1 h and then poured over ice (20 g). The resulting suspension of yellow-orange solid was acidified (litmus) with 5% HCl and extracted with EtOAc (4 X 25 mL). The solution was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (Universal, silica gel 60-40 micron, EtOAc:hexanes:AcOH, 1:1:0.01). Evaporation of the major fractions (tlc R_f = 0.73, silica gel, EtOAc:CH_3OH, 8:3) gave 175 mg of a yellow solid (58%). Recrystallization from EtOAc-hexanes afforded pure product with mp =162-164 °C (dec.) which was stored at -20 °C. IR (KBr, cm^{-1}) 3341, 2860 (br), 2112, 1675, 1580, and 1335; UV (CH_3OH) λ_{max} 249 nm (log ε = 4.26) and 367 nm (log ε = 4.24); ^{1}H-NMR (acetone-d_6) δ 8.91 (br s, 1H, COO<u>H</u>), 8.81 (d, 1H, J = 2.8 Hz, Ar<u>H</u> *ortho* to COOH), 8.19 (dd, 1H, J = 2.8 and 9.5 Hz, Ar<u>H</u> *para* to COOH), 7.34 (d, 2H, J = 8.5 Hz, Ar<u>H</u> *meta* to N_3), 7.02 (d, 2H, J = 8.5 Hz, ArH *ortho* to N_3), 6.90 (d, 1H, J =9.5 Hz, Ar<u>H</u> *meta* to COOH), 4.08 (br s, ~1H, N<u>H</u>), 3.43 (m, 2H), 2.79 (t, 2H), and 2.01 (buried m, ~2H); ^{13}C-NMR (acetone-d_6) 31.1, 32.8, 42.7, 109.6, 112.1, 119.8, 129.6, 130.3, 130.7, 138.5, 139.2, 155.7, 155.9, and 172.5; tlc (silica gel, $CHCl_3$:CH_3OH, 8:2, v/v) R_f = 0.75. HRMS (EI) calcd. for $C_{16}H_{15}N_5O_4$ 341.1126; found 341.1100. *Analysis*, $C_{16}H_{15}N_5O_4$; Calc. %: C, 56.30; H, 4.43. Found: C, 56.55; H, 4.27.

RESULTS AND DISCUSSION: *Synthesis.* The synthetic scheme used to prepare AzNPPB is shown in Figure 1. The starting materials for the convergent pathway were 1-bromo-3-phenylpropane, which was converted in two steps (9,10) into compound **III**, and 2-fluorobenzoic acid, which was transformed (8) into 2-fluoro-5-nitrobenzoic acid. The AzNPPB structure was confirmed by ^{1}H- and ^{13}C-NMR, IR, UV, high resolution MS, and elemental analysis. Two variations of the synthetic pathway to AzNPPB were developed (data not presented). In the first, compound **IV** was converted into the corresponding free base and the resulting phthalhydrazide was removed by filtration before the final coupling step. The second alteration involved deprotection of compound **III** with hydrazine, coupling of the resulting diamino product with 2-fluoro-5-nitrobenzoic acid, followed by conversion of the primary aryl amino group into the azide functionality.

Reversible inhibition. Using the $^{36}Cl^-$ efflux assay with resealed ghost preparations, both NPPB and AzNPPB were found to be reversible inhibitors of Cl^- efflux in the dark. The measured IC_{50} values

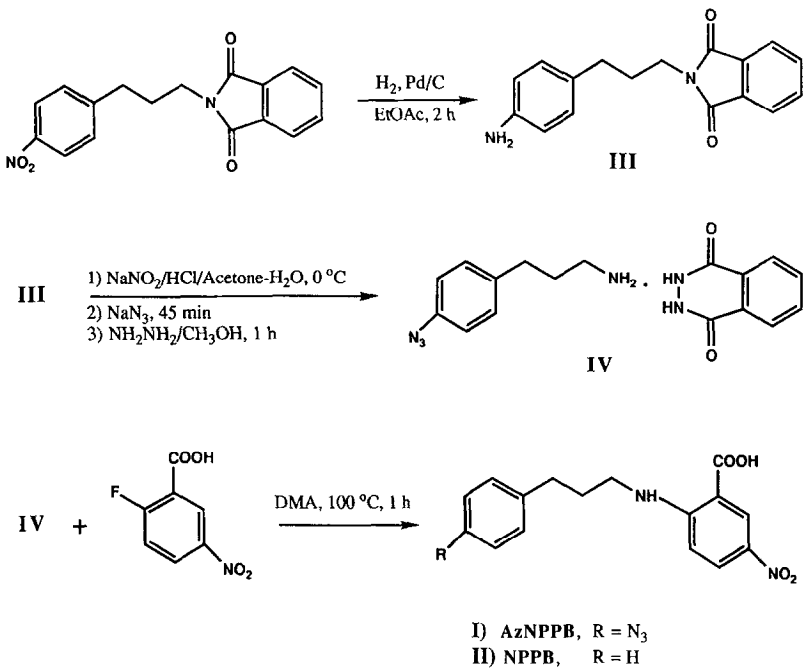

Figure 1. Synthetic route to AzNPPB (I) and structure of NPPB (II).

were 37 ±5 μM and 32 ±5 μM, respectively. The value for NPPB is in good agreement with the communicated report of ~30 μM (7). As assessed by ^{35}Cl-NMR methods (15) using leaky red cell membranes, NPPB and AzNPPB bind reversibly to the DNDS-sensitive Cl^- transport sites of band 3 protein. The apparent K_D values measured in 62.5 mM NaCl were 2.8 ± 0.5 μM and 2.1 ± 0.5 μM, respectively. The results of these experiments indicated that both NPPB and AzNPPB effectively and reversibly block Cl^- efflux in the chosen model system. The azido substituent of AzNPPB does not adversely affect these processes.

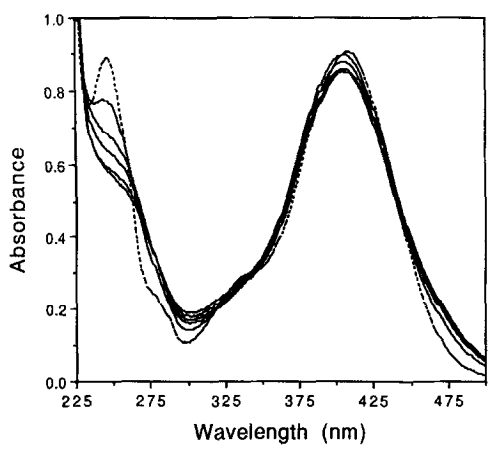

Figure 2. Photodecomposition of AzNPPB. A 45 μM solution of AzNPPB in efflux buffer was placed in a quartz cuvette 4 cm from a RPR "254" nm light source and irradiated at 21 °C for various time intervals. The ultraviolet-visible spectra were measured prior to irradiation (dashed line) and after 10, 30, 50, 120 and 500 s total exposure periods.

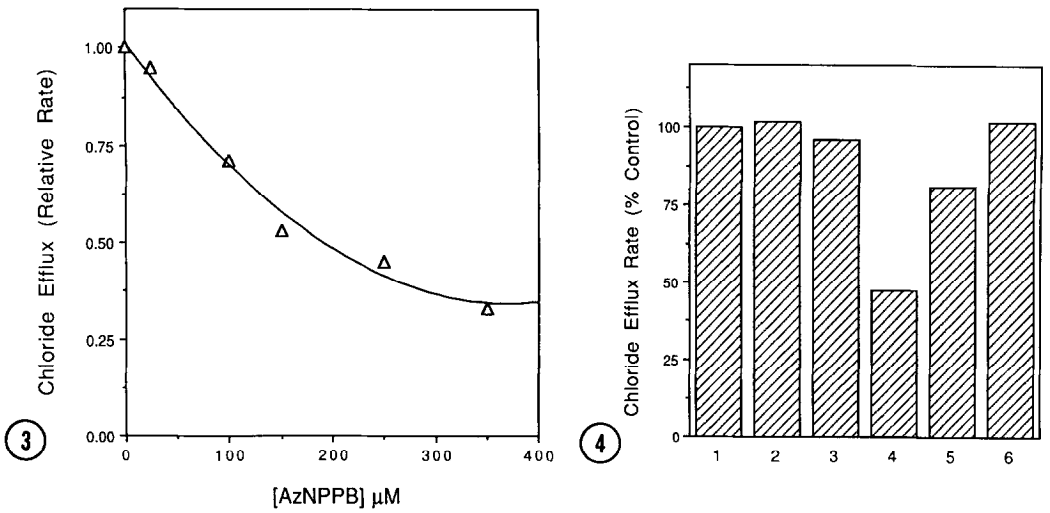

Figure 3. Concentration-dependent photoinactivation of Cl^- efflux in resealed ghost membranes. As described in *Methods*, resealed ghosts in efflux buffer were irradiated for 3 min with the concentrations of AzNPPB indicated above and then washed. Cl^- effluxes were measured using 100 μL of packed ghosts and rates were normalized to the control rate for ghosts irradiated in the absence of AzNPPB.

Figure 4. Photoaffinity control studies and protective effect of NPPB. As described in *Methods*, Cl^- efflux rates were measured with ghost membranes which had been treated for 5 min as follows: lane 1, irradiation with no additions; lane 2, incubation with 150 μM AzNPPB; lane 3, incubation with photoproducts from 200 μM AzNPPB; lane 4, irradiation with 150 μM AzNPPB; lane 5, irradiation with 150 μM AzNPPB and 75 μM NPPB; lane 6, irradiation with 150 μM AzNPPB and 150 μM NPPB. Cl^- efflux rates were expressed as % of the control rate for resealed ghosts which had been incubated without additions in the dark.

Photodecomposition. Solutions of AzNPPB in efflux buffer decompose when irradiated with a "254" nm UV source as evidenced by the absorption spectra shown in Figure 2. A half-life of 24 s at 21 °C was determined from the absorbance changes at 247 nm. It is likely that photodecomposition products arise from reactive nitrene intermediates.

Irreversible inhibition. The photolysis of solutions of resealed ghosts in efflux buffer (160 mM Cl^-) containing AzNPPB caused a concentration-dependent irreversible inactivation of Cl^- efflux which was not reversed by exhaustive washing (Figure 3). A 67% reduction in the rate of control Cl^- efflux was obtained with 350 μM AzNPPB. Higher concentrations of AzNPPB could not be tested for reasons of limited solubility. Photoinactivation was not increased when photolysis was performed in buffers containing 20 mM Cl^- (data not shown).

Photolysis controls. The results of control experiments presented in Figure 4 demonstrated that the photoinactivation of Cl^- efflux did not result from irradiation in the absence of AzNPPB (lane 1), the products of AzNPPB photodecomposition (lane 3), or noncovalently bound AzNPPB associated with the membranes (lane 2). Moreover, the 52% photoinactivation caused by 150 μM AzNPPB (lane 4) was partially prevented by 75 μM NPPB (lane 5) and completely prevented by 150 μM and 300 μM NPPB (lane 6). An additional control experiment demonstrated that AzNPPB underwent photodecomposition in the presence of 300 μM NPPB (data not shown). Therefore, the protective effect of NPPB was not simply a UV filtering effect.

Photoincorporation. An estimate of the extent of the photoincorporation of AzNPPB into erythrocyte ghost membranes was made by difference UV-Vis absorption spectroscopy. Difference UV

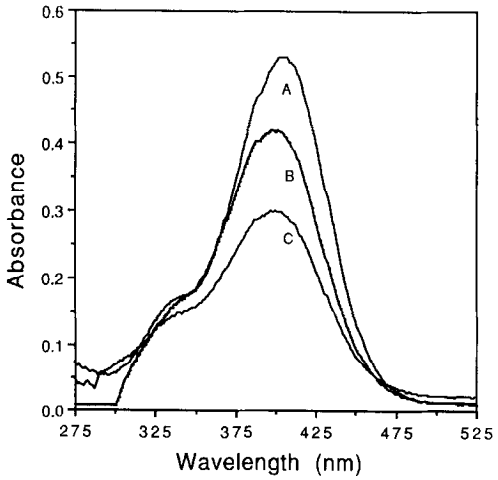

Figure 5. Photoincorporation of AzNPPB. As described in *Methods*, resealed ghosts were irradiated for 5 min with 150 μM AzNPPB in the absence or presence of 300 μM NPPB and then washed. Membranes were isolated by centrifugation and dissolved in efflux buffer diluted with an equal volume of 0.1% SDS in 0.1N NaOH. UV-Vis absorption spectra were obtained for these samples and a control solution of membrane proteins which had been incubated with 150 μM AzNPPB in the dark. Protein concentrations of all samples were adjusted to 1.7 mg/mL. The control spectrum was subtracted from the absorption spectra of the samples treated with AzNPPB alone (B) or with AzNPPB and NPPB (C). The absorption spectrum of 35 μM NPPB (A) is presented for comparison.

data were obtained by subtraction of a control absorption spectrum of ghost membranes incubated with AzNPPB in the dark from spectra of resealed ghosts which had been irradiated with 150 μM AzNPPB in the absence (Figure 5, B) or presence of 300 μM NPPB (Figure 5, C). Control experiments were performed in which a spectrum of photolyzed ghost membranes (nothing added) was subtracted from spectra of ghost membranes which had been incubated with: (i) 150 μM AzNPPB in the dark; (ii) photoproducts from 200 μM AzNPPB; or (iii) 300 μM NPPB. None of these produced positive difference absorption. Using the absorbance values at 401 nm obtained from Figure 5, an ε_{401nm} = 15,610 based on NPPB absorption, and an approximate concentration of band 3 sites equal to 4.5 μM (16), a calculated value of approximately 6 molecules of AzNPPB per band 3 site were incorporated during photolysis without NPPB. Similarly, it was determined that approximately 4 molecules per site bound when 300 μM NPPB was present during irradiation. The basis for these estimates is that membranes will show positive difference long wavelength absorption only if the substituted 2-amino-5-nitrobenzoic acid chromophore is covalently attached. The exact membrane sites involved or the position of attachment on the ring originally containing the reactive aryl azide group have little effect on this absorption. These assumptions were supported by the measured UV spectrum of 2-amino-5-nitrobenzoic acid which shows λ_{max} =383 nm; ε =13,000.

Conclusions. This communication establishes the synthesis and characterization of AzNPPB, the first photoaffinity reagent based on the structure of NPPB. The synthesis can be readily modified to prepare a potentially more effective tetrafluoroarylazido analog (17); this preparation is currently in progress. Moreover, the preparation of radiolabeled analogs of 2-fluoro-5-nitrobenzoic acid, one of which has been described (18), will enable radiolabeled versions of AzNPPB and future analogs to be prepared from a single precursor. This work too is presently in progress.

Using erythrocyte ghost membranes as a model system, we have employed $^{36}Cl^-$ efflux and ^{35}Cl - NMR methods to demonstrate that AzNPPB reversibly blocks Cl^- efflux as effectively as NPPB. Unlike

NPPB, AzNPPB is photochemically labile (Figure 2), and irradiation of ghost membranes with AzNPPB results in photoinactivation (Figure 3) and photoincorporation (Figure 5). The high level of measured AzNPPB incorporation may be the result of nonspecific binding, possibly including photolabeling of membrane lipids. NPPB can completely protect membrane sites from AzNPPB photoinhibition (Figure 4); and NPPB partially prevents photoincorporation (Figure 5).

AzNPPB may be a useful structure probe for studies on the mechanism of Cl^- transport in ghost membranes. Aryl azide **I** may play a complementary role to the previously described reagent NAP-Taurine (19), a photoaffinity probe for the modifier site of the erythrocyte anion transport system. Moreover, the AzNPPB structure should be the basis for highly useful photoprobes for many of the systems (4,7) that have been shown to be sensitive to NPPB.

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