5′-(*p*-Fluorosulfonylbenzoyl)-2′(or 3′)-(methylanthraniloyl)adenosine, Fluorescent Affinity Labels for Adenine Nucleotide Binding Sites: Interaction with the Kinase Active Site of the Receptor for Epidermal Growth Factor†

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ABSTRACT: We have found that the epidermal growth factor (EGF) receptor kinase can utilize the fluorescent ATP derivative, methylanthraniloyl ATP, as a substrate. On the basis of this observation, together with our previous studies that showed that 5′-(*p*-fluorosulfonylbenzoyl)adenosine (5′-FSBAdo) is a highly specific affinity label for the ATP site of the kinase domain of the EGF receptor, we prepared new derivatives of 5′-FSBAdo, 5′-(*p*-fluorosulfonyl)-2′(or 3′)-(methylanthraniloyl)adenosine (FSBMantAdo), as fluorescent affinity labels for adenine nucleotide binding sites, and in particular for the ATP site of the EGF receptor. The two products were purified by HPLC and were characterized by UV−Vis absorbance spectroscopy, mass spectrometry, nuclear magnetic resonance spectroscopy, and fluorescence spectroscopy. Incubation of membrane vesicles containing the EGF receptor with either the 2′ or 3′ derivative resulted in irreversible inhibition of the receptor kinase activity, as assessed by autophosphorylation assays. Preincubation of vesicles with AMP imidodiphosphate (AMPPNP), a hydrolysis-resistant ATP analog, prior to treatment with FSBMantAdo resulted in the protection of the receptor kinase activity from FSBMantAdo inactivation. Steady state fluorescence spectra (with excitation at 360 nm) revealed a blue shift in the emission maximum of partially purified FSBMantAdo-labeled receptor (426 nm), as compared with the emission maximum of free FSBMantAdo (441 nm) in aqueous solution, suggesting that the receptor-bound label is in a relatively low polarity environment. These studies show that FSBMantAdo is a specific affinity label for the ATP site of the EGF receptor. FSBMantAdo may also prove useful as a fluorescent affinity label for other ATP binding sites.

The binding of EGF[1] to its cell surface receptor activates a protein kinase (Carpenter *et al.*, 1978, 1979) specific for tyrosyl residues (Ushiro & Cohen, 1980) that phosphorylates the receptor (Cohen *et al.*, 1980) as well as other intracellular substrates. Studies using the adenine nucleotide affinity label 5′-FSBAdo showed that the EGF receptor and the EGF-stimulable Tyr kinase reside within the same molecule (Buhrow *et al.*, 1982, 1983), so that ligand binding which activates the kinase results in autophosphorylation. The molecular identity of the receptor and the kinase was confirmed and expanded by the cloning and sequencing of cDNA to the receptor, revealing an 1186-residue protein with a kinase-homologous domain in the carboxyl terminal half of the molecule (Ullrich *et al.*, 1984). Edman sequencing of the labeled tryptic fragment from 5′-FSBAdo-modified receptor revealed the unique site of affinity labeling to be Lys721 (Russo *et al.*, 1985).

In addition to stimulating autophosphorylation and phosphorylation of other intracellular substrates, the binding of EGF has been shown to trigger receptor dimerization (Böni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987; Cochet *et al.*, 1988; Fanger *et al.*, 1989). Autophosphorylation of the receptor leads to other protein−protein interactions, as well, through association of the SH2 domains [reviewed in Pawson and Gish (1992) and Fry *et al.* (1993)] or PTB domains (Kavanaugh & Williams, 1994; Blaikie *et al.*, 1994; Kavanaugh *et al.*, 1995) of various cytoplasmic proteins with sites of Tyr phosphorylation on the receptor. In order to probe spectroscopically protein−protein interactions of the receptor, such as EGF binding, receptor dimerization, and interaction with SH2 or PTB domain proteins, we have prepared two fluorescent nucleotide affinity labels designed to covalently modify the ATP binding pocket in the active site of the kinase domain of the receptor.

Fluorescent nucleotide affinity labels have proven useful in a wide range of structure−function studies of enzymes [reviewed in Colman (1983) and Colman *et al.* (1990)]. Since their introduction fifteen years ago, the fluorescent 5′-fluorosulfonylbenzoyl derivatives of 1,N^6-ethenoadenosine

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[1] Abbreviations: DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FSB, fluorosulfonylbenzoyl; Mant, methylanthraniloyl; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; PTB, phosphotyrosine binding domain; $NaDodSO_4$, sodium dodecyl sulfate; SH2, src homology (domain) 2.

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(Likos & Colman, 1981) and 2-aza-1,N^6-ethenoadenosine (Craig & Hammes, 1980) have gained the widest application. These derivatives have been used to study characteristics such as amino acid conformation in the nucleotide binding site (Tomich & Colman, 1985) and, in conjunction with other fluorescent labels in energy transfer experiments, to determine distances between two sites (Jacobson & Colman, 1983). A more recent addition to the ethenoadenosine family is 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine, which has been prepared as the 2′,5′-bisphosphate (Bailey & Colman, 1985) and as the 5′-diphosphate (DeCamp & Colman, 1989).

A characteristic of the ethenoadenosine family of fluorescent nucleotide affinity labels is the modification of the adenine ring, limiting their use in enzymes in which the unmodified adenine moiety is essential for high-affinity binding. In the case of the EGF receptor, modification of the adenine ring greatly reduces the affinity of adenine nucleotides (Vogel *et al.*, 1986). We therefore focused on fluorescent groups that could be linked to the ribose moiety.

One fluorescent group with demonstrated utility for probing nucleotide binding sites when linked to ATP or GTP *via* the 2′ or 3′ hydroxyl of the ribose moiety is the methylanthraniloyl (Mant) group. For example, MantGTP or MantATP has been used to monitor a conformational change in $p21^{N-ras}$ (Neal *et al.*, 1990), to assess the kinetics of nucleotide interaction with the S1 fragment of myosin (Woodward *et al.*, 1991), to measure distances within yeast mitochondrial F_1-ATPase by fluorescence resonance energy transfer (Divita *et al.*, 1993), and to study the mechanism of GTP hydrolysis by $p21^{N-ras}$ (Moore *et al.*, 1993). To test whether the Mant group could be accommodated in the ATP site of the EGF receptor, we synthesized MantATP (Hiratsuka, 1983) and tested the ability of the receptor to utilize it as a substrate. The successful result of that study led us to synthesize and characterize 5′-(*p*-fluorosulfonylbenzoyl)-2′-(or 3′)-(methylanthraniloyl)adenosine, new fluorescent adenine nucleotide affinity labels with unmodified adenine rings. We further describe the specific modification of the kinase active site of the EGF receptor with these reagents and the fluorescent properties of the 5′-FSB-3′-MantAdo adduct.

EXPERIMENTAL PROCEDURES

Materials

Membrane vesicles from A431 cells were prepared by a modification of the method of Cohen *et al.* (1982). [For details, see the supporting information for Rousseau *et al.* (1995).] Murine EGF was prepared as previously described (Savage & Cohen, 1972). MantATP was synthesized as previously described (Hiratsuka, 1983).[2] Deuterated methanol (MeOD), D_2O, and dry deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Aldrich. 5′-FSBAdo and AMPPNP were purchased from Sigma, and methylisatoic anhydride was purchased from Aldrich. The detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) was purchased from Fluka. Dimethylformamide (DMF) and triethylamine were purchased from Pierce. [γ-^{32}P]ATP was purchased from Dupont-NEN. Horseradish peroxidase-linked goat anti-mouse serum and horseradish peroxidase-linked donkey anti-rabbit serum were purchased from Amersham Life Sciences. The phosphotyrosine antibody, RC20H, was purchased from Transduction Laboratories. All other chemicals were of at least reagent grade. EGF receptor antiserum was a generous gift from Dr. Graham Carpenter (Vanderbilt University).

Methods

MantATP Phosphorylation Assays. EGF receptor kinase activity was measured using MantATP as a substitute for [γ-^{32}P]ATP in a standard autophosphorylation assay. A431 membrane vesicles were used as the source of EGF receptor. For each autophosphorylation assay sample, 3 μL of 0.1 mg/ml murine EGF [or 20 mM HEPES buffer (pH 7.4) for control samples] was incubated for 10 min at room temperature with 50 μL of a suspension of A431 vesicles prepared by a 20-fold dilution of vesicle stock in 20 mM HEPES and 40 mM Na_2HPO_4 buffer (pH 7.4). Autophosphorylation was initiated by addition of the sample to 5 μL of 10× phosphorylation buffer (previously chilled on ice) to give a final phosphorylation buffer concentration of 25 mM HEPES (pH 7.4), 10 μM sodium orthovanadate, 1 mM $MnCl_2$, 5 mM $MgCl_2$, and either 0, 3, 15, 30, 60, 90, 120, or 150 μM MantATP. The autophosphorylation reaction was allowed to proceed for 2 min on ice and was then terminated by addition of 4× electrophoresis sample buffer [1× = 62.5 mM Tris (pH 6.8), 2% $NaDodSO_4$, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue] followed by heating to 95 °C for 5 min. Samples were analyzed using $NaDodSO_4$−polyacrylamide gel electrophoresis (Laemmli, 1970), followed by electrophoretic transfer of proteins to nitrocellulose (Micron Separations, Westboro, MA) using a TE77 SemiPhor transfer unit (Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions, with the exception that the transfer buffer used was 48 mM Tris base, 39 mM glycine, 0.037% (v/v) $NaDodSO_4$, and 20% methanol. Transferred proteins were immunostained using the horseradish peroxidase-conjugated, phosphotyrosine monoclonal antibody RC20H (Transduction Laboratories, Lexington, KY) and visualized by enhanced chemiluminescence using the Renaissance Western Blot Chemiluminescence Reagent kit (DuPont-NEN).

Synthesis of 5′-(p-Fluorosulfonyl)-2′(or 3′)-(methylanthraniloyl)adenosine. In a 1 mL glass reaction vial, 0.05 mmol (25.5 mg) of 5′-FSBAdo was added to 0.10 mmol (17.7 mg) of methylisatoic anhydride. The reactants were dissolved in 0.93 mL of DMF, and the reaction vial was sealed with a Teflon cap. Argon was layered over the reaction mixture, and 0.05 mmol (7 μL) of triethylamine was added to initiate the reaction, which was then stirred overnight at room temperature. Products of the reaction were initially identified by HPLC using a 4.6 × 220 mm Dynamax 300 Å C-18 column with a 56 min linear gradient from 100% Milli-Q H_2O (solvent A) to 100% methanol (solvent B) at a flow rate of 1 mL/min. The solvent delivery system consisted of two Waters 510 pumps controlled by a Maxima

[2] Hiratsuka (1983) reported that essentially exclusively the 3′ derivative was obtained; however, Cremo *et al.* (1990) reported that MantADP synthesized under the same conditions yielded 65% 3′ and 35% 2′ isomers. It is not unlikely that the different results could be due to slow interconversion between the two isomers (*vide infra*). Because our purpose was to ascertain whether the Mant group in either position could be accommodated by the EGF receptor, we employed the MantATP synthesized by the method of Hiratsuka (1983) without any attempt to resolve isomers.

820 workstation and a Waters 490e programmable multi-wavelength detector monitoring absorbance at 220, 260, and 350 nm.

HPLC Purification of FSBMantAdo. A portion of the crude reaction mixture (0.30 mL) was added to 10 mL of Milli-Q H_2O, which resulted in precipitation of the products. The resulting suspension was filtered through a 0.45 μm Acrodisc syringe filter (Gelman Science), and the filtrate was discarded. The filter was then repeatedly washed by recycling 6 mL of HPLC grade methanol (Baxter) through the filter to dissolve the products which had been collected by filtration. Prior to separation by HPLC, 4 mL of H_2O was added to the 6 mL methanolic solution and the entire sample was injected. This procedure was repeated until the entire crude reaction mixture had been processed.

A preparative scale HPLC separation was employed, using a 21.4 × 250 mm Dynamax 300 Å C-18 column with Milli-Q H_2O (solvent A) and methanol (solvent B) as mobile phases and a 27 min linear gradient of 50 to 100% solvent B at a flow rate of 15 mL/min. The solvent delivery system consisted of a Waters 600E Multisolvent Delivery System and a Waters 486 variable-wavelength detector used to monitor absorbance at 220 nm. Pooled peaks of HPLC-purified samples were lyophilized to dryness, redissolved in dry DMF, layered with argon, and stored at −20 °C (or −70 °C) until needed.

UV−Vis Spectroscopy of FSBMantAdo. Spectrophotometric analysis of HPLC-purified fractions of the FSBMantAdo synthesis and of starting materials was performed on a Cary 1E UV−Vis spectrophotometer. Aliquots of the FSBMantAdo stocks in DMF were added to 20 mM HEPES (pH 7.4) for spectral measurements, and absorbance spectra were obtained from 200 to 450 nm for the five major peaks from the HPLC purification of the FSBMantAdo synthesis. Baselines were determined using 20 mM HEPES (pH 7.4) with aliquots of DMF added to equal the DMF in aliquots of FSBMantAdo stocks.

Mass Spectrometry of FSBMantAdo. HPLC-purified FSBMantAdo in a matrix of 20% 3-nitrobenzyl alcohol and 20% polyethylene glycol 200/400/600 in DMSO was analyzed by liquid secondary ionization (LSI) mass spectrometry on a Kratos Koncept II HH mass spectrometer in the Vanderbilt University Mass Spectrometry Resource Center.

Fluorescence Emission Measurements of FSBMantAdo in Solution. Samples of FSBMantAdo for fluorescence emission were prepared by dissolving the FSBMantAdo in DMF, preparing dilute stocks in DMF, and then adding 2 μL aliquots of each DMF stock solution to 998 μL of 20 mM HEPES (pH 7.4) to give final concentrations of 2.5, 1.0, and 0.5 μM. Samples at 0.25 and 0.1 μM were prepared by dilution of the 0.5 μM sample in 20 mM HEPES (pH 7.4). Stock concentrations were determined by absorption at 360 nm, using $\epsilon = 5800\ M^{-1}$ (Hiratsuka, 1983). Emission spectra of varying concentrations of FSBMantAdo were obtained on an SLM 8100 fluorometer from 380 to 530 nm with an excitation wavelength of 360 nm.

Nuclear Magnetic Resonance (NMR) Analysis of HPLC-Purified FSBMantAdo. The solvent was removed under vacuum from the two HPLC-purified fractions identified as containing FSBMantAdo using a Speed Vac Concentrator (Savant Instruments). The samples were redissolved in deuterated methanol (MeOD), followed by the addition of D_2O to give a 1:1 dilution of D_2O−MeOD. Each sample was again reduced to dryness, and the procedure was repeated four times to remove H_2O which could interfere with the resolution of the spectrum. The samples were then redissolved in dry deuterated DMSO-d_6, and one-dimensional and COSY spectra were obtained on a Bruker AM-400, 400 MHz instrument.

Inhibition of EGF Receptor Kinase Activity by 5′-FSB-3′-MantAdo and Protection by AMPPNP. The inhibition of EGF receptor kinase activity by 5′-FSB-3′-MantAdo and the ability of AMPPNP to protect against receptor inhibition due to FSBMantAdo labeling were determined by examining the time course and concentration response of inhibition. A431 membrane vesicles (130 μL, 10 mg/ml) were diluted to 1.2 mL with 20 mM HEPES (pH 7.4) and 5 mM $MgCl_2$, and aliquots of the diluted membrane vesicles (575 μL) were then incubated in the absence or presence of 2 mM AMPPNP (reaction volume = 639 μL) to determine the specificity of the FSBMantAdo reaction for the ATP binding site of the EGF receptor. After 10 min at room temperature, 19 μL samples were removed in triplicate, washed, and assayed as described below in order to serve as the zero time points for the determination of maximal receptor kinase activity. Modification with FSBMantAdo was accomplished by the addition of 95 μL of each vesicle preparation to 5 μL of DMF containing 5′-FSB-3′-MantAdo to yield final concentrations of 10, 50, or 100 μM 5′-FSB-3′-MantAdo. For control reactions, each vesicle preparation was incubated with 5 μL of either water or DMF to determine the effect of the modification time and the DMF solvent on receptor kinase activity. All reactions were performed at room temperature. At specified times, aliquots (20 μL, to compensate for the slight dilution by the added reagent) were removed from each reaction mixture and the vesicles were washed by the addition of 1 mL of 20 mM HEPES (pH 7.4). The vesicles were centrifuged for 5 min at 16000*g* at 4 °C, and the supernatant was removed. The pellet was resuspended by vortexing in 45 μL of 20 mM HEPES (pH 7.4) and 0.01 mg/mL EGF. The samples were incubated for 10 min on ice, and phosphorylation assays were initiated by the addition of 5 μL of 10× phosphorylation buffer to yield final concentrations of 20 mM HEPES (pH 7.4), 10 μM sodium vanadate, 1 mM $MnCl_2$, 5 mM $MgCl_2$, and 20 μM ATP with 1.0 μCi of [γ-^{32}P]ATP (3×10^3 Ci/mmol) per assay. The phosphorylation reaction mixtures were incubated for 3 min on ice, the reactions quenched with 4× electrophoresis sample buffer, and then the mixtures incubated at 95 °C for 4 min. The phosphorylated proteins were separated by $NaDodSO_4$−polyacrylamide gel electrophoresis on a 6% gel (Laemmeli, 1970), and the extent of receptor kinase inhibition was assessed by autoradiography of the dried gel and then quantified by phosphorimage analysis using a Molecular Dynamics model 445Si phosphorimager.

Wheat Germ Lectin Purification of FSBMantAdo-Labeled EGF Receptor. A431 vesicles, 400 μL, in 2.64 mL of 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, and 1 mM $MnCl_2$ were incubated in the presence of 100 μM 5′-FSB-3′-MantAdo for 60 min at room temperature. The vesicles were washed with 1 mL of 20 mM HEPES (pH 7.4) and centrifuged for 30 min at 47800*g* at 4 °C, after which the supernatant was discarded. The pelleted vesicles were solubilized in 4 mL of 20 mM HEPES (pH 7.4), 1% $C_{12}E_8$, 10% glycerol, 10 mM EGTA, 10 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mg/mL aprotinin, and 0.1 mg/mL leupeptin followed by

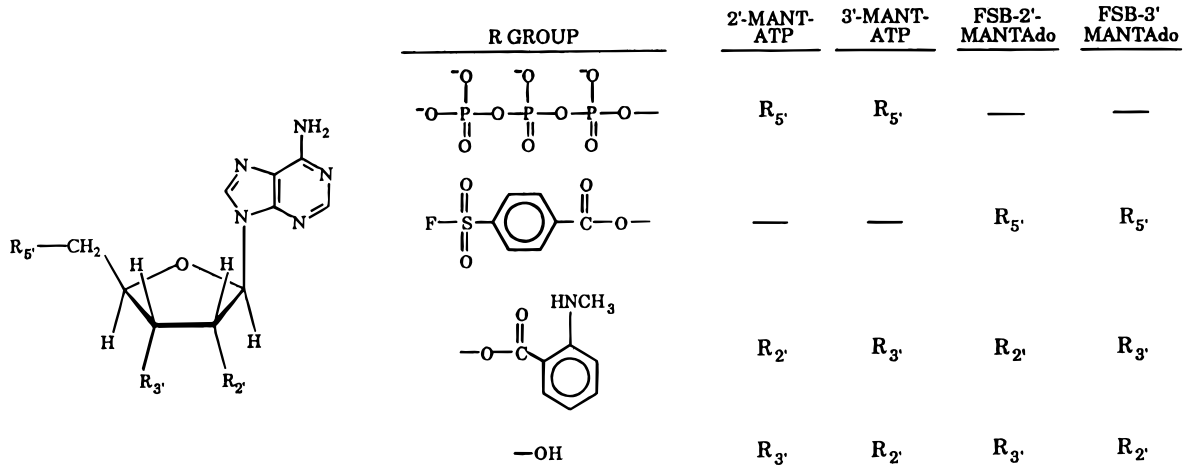

FIGURE 1: Structures of the methylanthraniloyl (Mant) adenosine (Ado) derivatives used in this study.

centrifugation for 30 min at 100000*g* at 4 °C. After the supernatant was adjusted to a final concentration of 10 mM $MgCl_2$, the sample was added to hydrated wheat germ agglutinin (WGA)−agarose (400 μL of packed beads) and the resulting suspension was rocked overnight at 4 °C. The samples were centrifuged and washed four times with 20 mM HEPES (pH 7.4), 1% $C_{12}E_8$, 10% glycerol, 100 mM NaCl, and 10 mM $MgCl_2$. Bound glycoproteins including the EGF receptor were eluted with 20 mM HEPES (pH 7.4), 1% $C_{12}E_8$ detergent, 10% glycerol, and 100 mM NaCl, containing 3 mM *N*,*N*′,*N*″-triacetylchitotriose. Samples were stored at −70 °C until they were needed.

Fluorescence Emission of FSBMantAdo-labeled EGF Receptor. Emission spectra of WGA−agarose-purified FSBMantAdo-labeled receptor (200 μL) were obtained on an SLM 8100 fluorometer. Spectra were obtained by exciting the FSBMantAdo moiety at 360 nm and measuring fluorescence emission between 380 and 530 nm.

RESULTS AND DISCUSSION

Previous studies from this laboratory have shown that 5′-FSBAdo functions as an efficient affinity label for the EGF receptor kinase (Buhrow *et al.*, 1982, 1983), labeling it at a unique site, Lys721 (Russo *et al.*, 1985). The 5′-*p*-fluorosulfonylbenzoyl moiety was, therefore, a good candidate for the reactive group for a fluorescent affinity label for the ATP site of the receptor. Given the previous observation that even minor modification of N^6 of the adenine ring of ATP results in a dramatic decrease in the affinity of the receptor for the modified nucleotide (Vogel *et al.*, 1986), we explored modifying the free hydroxyl groups of the ribose ring of 5′-FSBAdo with Mant, a fluorescent group successfully employed for probing adenine and guanine nucleotide sites (Neal *et al.*, 1990; Woodward *et al.*, 1991; Divita *et al.*, 1993; Moore *et al.*, 1993).

As a test of the ability of the receptor to recognize Mant-modified adenine nucleotides, MantATP[2] (Figure 1) was synthesized by the method of Hiratsuka (1983) and was employed in EGF receptor autophosphorylation assays in place of ATP. As shown in Figure 2, MantATP was able to support EGF-stimulated autophosphorylation of the receptor at a level comparable to that of ATP. Having found that the Mant group as well as the 5′-*p*-fluorosulfonylbenzoyl moiety could be accommodated by the EGF receptor, we prepared FSBMantAdo (Figure 1).

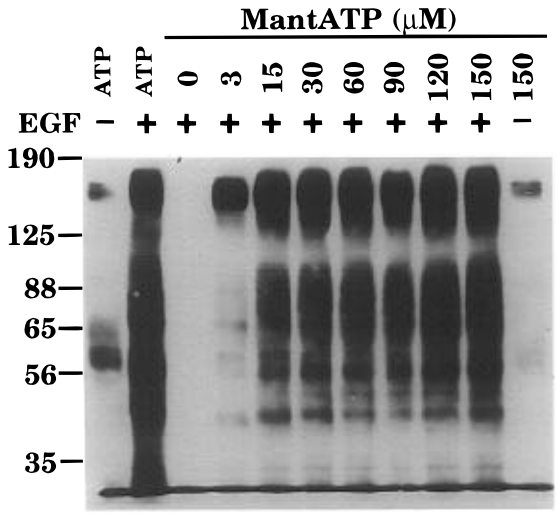

FIGURE 2: MantATP as a substrate for EGF receptor kinase activity. Membrane vesicles from A431 cells were subjected to autophosphorylation assays in the absence or presence of EGF and in the absence of nucleotide or in the presence of ATP (15 μM) or MantATP, at the concentrations indicated in the figure. Samples were subjected to electrophoresis in $NaDodSO_4$−polyacrylamide gels, were transferred to nitrocellulose, and were immunostained with RC2OH phosphotyrosine monoclonal antibody, as detailed in Methods.

The synthesis of MantATP from methylisatoic anhydride and ATP had been carried out in aqueous solution at pH 9.6 (Hiratsuka, 1983), conditions incompatible with the fluorosulfonylbenzoyl group. Under the anhydrous conditions that we employed for the synthesis of FSBMantAdo from FSBAdo and methylisatoic anhydride, two isomers were formed, which were purified from the reaction mixture by HPLC on a C-18 column (see Methods). Five peaks unique to the reaction mixture were initially collected for analysis. Absorbance spectral analysis from 200 to 450 nm of the five collected HPLC-purified fractions identified those fractions that possessed absorbance spectra similar to those previously published for Mant nucleotide derivatives. The ratio of the 360 nm peak absorbance to the 260 nm peak absorbance gives information on the number of methylanthraniloyl groups attached to the ribose ring. Of the five peak fractions that were found to absorb at 260 and 360 nm, two possessed a 260:360 nm absorbance ratio of approximately 4:1 (Figure 3), which is consistent with the presence of one group attached to each adenosine molecule, as previously described for MantATP (Hiratsuka, 1983).

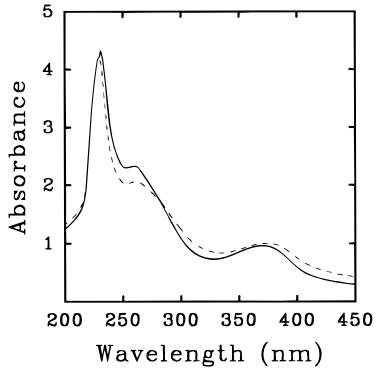

FIGURE 3: Spectrophotometric analysis of HPLC-purified isomers of FSBMantAdo. Aliquots of HPLC-purified FSBMantAdo isomers in DMF were added to 20 mM HEPES (pH 7.4), and absorbance spectra were obtained from 200 to 450 nm. The spectra shown are corrected by subtracting background obtained from a sample consisting of the same concentration of DMF in the HEPES buffer. The dashed line corresponds to the 2′ isomer, and the solid line corresponds to the 3′ isomer.

To characterize further these two compounds, liquid secondary ionization mass spectra were acquired. An accurate mass of 587.137 (MH^+) was obtained for both compounds, which is within ~2 ppm of the mass calculated (587.136) for the protonated form of FSBMantAdo. Mass spectral data also yielded a chemical composition for the compounds consistent with (protonated) FSBMantAdo, $C_{25}H_{24}N_6O_8FS$.

In order to determine which isomer corresponded to which of the HPLC peaks, the two fractions were analyzed by NMR spectroscopy and the results were compared with those of Cremo *et al.* (1990), resulting in the identification of the earlier-eluting HPLC fraction as the 2′ isomer and the later-eluting HPLC fraction as the 3′ isomer. Both NMR spectra revealed some contamination with the other isomer. This was especially prominent in the 2′ isomer sample. Because the two compounds were separated well by HPLC, it is likely that interconversion between the isomers occurred after separation. Such interconversion has been reported for Mant nucleosides and nucleotides under various conditions (Hiratsuka, 1983; Cremo *et al.*, 1990; Rensland *et al.*, 1991; Moore *et al.*, 1993). Interestingly, once the samples were dissolved in dry DMSO-d_6 for NMR analysis, no further interconversion was observed upon storage.

Fluorescence emission spectra of a range of concentrations of 5′-FSB-2′-MantAdo and 5′-FSB-3′-MantAdo in 20 mM HEPES at an excitation wavelength of 360 nm were acquired. Data for the 3′ isomer are shown in Figure 4. The emission maximum of the free reagent in aqueous solution is at 441 nm, consistent with previous reports for Mant-derivatized adenine nucleotides (Hiratsuka, 1983; Cremo *et al.*, 1990). Under the conditions tested, the saturating concentration of 5′-FSB-3′-MantAdo appears to fall between 1 and 2.5 μM, as judged from the nonlinearity of the fluorescence emission above 1 μM, together with a slight (~2 nm) red shift of the emission maximum of the 2.5 μM sample.

Fluorescent labeling of the receptor was assessed by incubating A431 cell membrane vesicles with 100 μM 5′-FSB-3′-MantAdo for 60 min at room temperature, solubilizing the vesicles in buffer containing 1% $C_{12}E_8$, partially purifying the labeled receptor on WGA−agarose, and examining the partially purified, labeled receptor by fluorescence emission spectroscopy. Comparison of the fluorescence emission maximum for 5′-FSB-3′-MantAdo in solution with that for the SB-3′-MantAdo moiety covalently attached to the receptor (Figure 5) reveals a 15 nm blue shift from 441 to 426 nm, suggestive of a relatively nonpolar environment at the site at which the EGF receptor is labeled with FSBMantAdo.

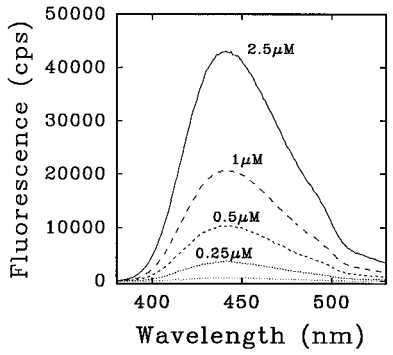

FIGURE 4: Fluorescence emission of 5′-FSB-3′-MantAdo in aqueous solution. Emission spectra (excitation at 360 nm) are shown for 5′-FSB-3′-MantAdo at nominal concentrations of 0.1 (lowest trace, unlabeled), 0.25, 0.5, 1.0, and 2.5 μM. The emission does not increase linearly between 1.0 and 2.5 μM, due to the limited solubility of 5′-FSB-3′-MantAdo in aqueous solution.

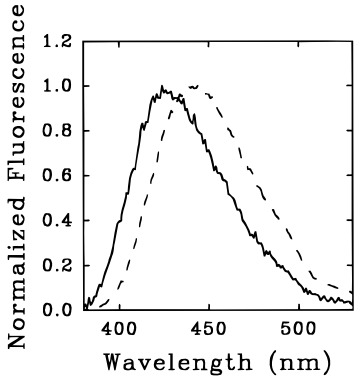

FIGURE 5: Fluorescence emission of 5′-FSB-3′-MantAdo-labeled EGF receptor. A431 membrane vesicles were modified with FSBMantAdo and partially purified on WGA−agarose, as described in the text. Excitation was at 360 nm, and the emission maximum was observed at 426 nm (solid line). The spectrum with the peak at 441 nm (dashed line) is free 5′-FSB-3′-MantAdo in aqueous solution (as in Figure 4), shown to illustrate the blue shift that occurs upon reaction of FSBMantAdo with the receptor.

Previous affinity labeling studies of the EGF receptor showed that 5′-FSBAdo inhibits the kinase activity of the receptor (Buhrow *et al.*, 1982, 1983) by specifically reacting with Lys721 in the ATP binding site (Russo *et al.*, 1985). To test whether FSBMantAdo also modifies the ATP site, reaction of 5′-FSB-3′-MantAdo with the EGF receptor was followed by measuring the inhibition of EGF receptor kinase activity, as assessed by EGF-stimulated receptor autophosphorylation with [γ-^{32}P]ATP (Figure 6). Incubation of EGF receptor with 50 or 100 μM 5′-FSB-3′-MantAdo for 1 h at room temperature gave approximately 80% inhibition of autophosphorylation activity, comparable to the inhibition observed with 5′-FSBAdo (Buhrow *et al.*, 1982; Russo *et al.*, 1985). In these experiments, irreversible inhibition was confirmed by the inability to remove the inhibitory effects of FSBMantAdo by washing the samples in 20 mM HEPES (pH 7.4).

The specificity of 5′-FSB-3′-MantAdo modification of the EGF receptor was further tested by measuring the effect of

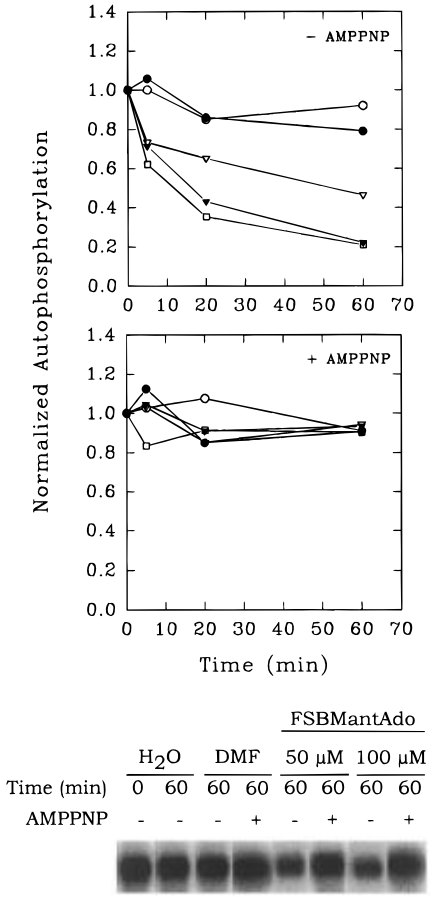

FIGURE 6: Irreversible inhibition of EGF receptor kinase activity by 5′-FSB-3′-MantAdo and protection from inhibition by AMPPNP. Membrane vesicles derived from A431 cells were incubated in the absence or presence of AMPPNP at room temperature for 10 min, after which samples corresponding to the 0 time point were removed to normalize the receptor kinase activity for the assay. Aliquots of the vesicle preparations were then treated with various concentrations of 5′-FSB-3′-MantAdo in DMF (10 μM, open triangles; 50 μM, filled triangles; and 100 μM, open squares). For control reactions, vesicle preparations were incubated with either water (open circles) or DMF (filled circles). At the indicated times (5, 20, or 60 min), aliquots (20 μL) were removed and the vesicles were washed by the addition of 1 mL of 20 mM HEPES (pH 7.4) followed by centrifugation. The pellets were resuspended and subjected to autophosphorylation assays, as described in the text. Samples were separated in 6% $NaDodSO_4$−polyacrylamide gels, and the resulting dried gels were subjected to autoradiography followed by phosphorimage analysis of the band corresponding to the EGF receptor in each lane. The gel shows the extent of EGF receptor phosphorylation following incubation of vesicles in the absence (0 and DMF controls) or presence of 50 or 100 μM FSBMantAdo for 60 min and the protection afforded by a 10 min preincubation with 2 mM AMPPNP. The graphs represent the averages of three independent experiments. The gel corresponds to the data from one of these independent experiments.

AMPPNP on kinase inhibition by 5′-FSB-3′-MantAdo. AMPPNP, a hydrolysis-resistant ATP analog (Yount *et al.*, 1971), has been shown previously to protect the EGF receptor from modification with 5′-FSBAdo (Buhrow *et al.*, 1982). The data in Figure 6 show that AMPPNP also protects the EGF receptor autophosphorylation activity from inactivation by 5′-FSB-3′-MantAdo.

The kinase inhibition data and the AMPPNP protection data together provide convincing evidence that FSBMantAdo specifically modifies the ATP site of the EGF receptor. The magnitude of the blue shift observed suggests that all of the receptor-associated Mant is in a relatively nonpolar environment, arguing against nonspecific labeling of surface amino (or other reactive) groups. These data suggest that 5′-FSB-3′-MantAdo, like 5′-FSBAdo, which we have previously shown labels at detectable levels only one residue of the EGF receptor, Lys721 (Russo *et al.*, 1985), reacts at a single site in the ATP binding site, presumably also modifying Lys721. The properties of FSBMantAdo suggest that it will prove useful in studies of the interaction of the EGF receptor with other proteins involved in the EGF-triggered signaling pathway and that it will likely prove useful as a fluorescent probe of ATP sites in a range of other enzymes, as well.

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