Fluorescent Substrate Analogue for Adenosine Deaminase: 3′-*O*-[5-(Dimethylamino)naphthalene-1-sulfonyl]adenosine†

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ABSTRACT: The synthesis of the fluorescent derivative of adenosine, by reaction with 5-(dimethylamino)naphthalene-1-sulfonyl chloride in dry pyridine at low temperature, yielding 3′-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl]adenosine (3′-*O*-dansyladenosine), is here described. 3′-*O*-Dansyladenosine is partially soluble in water ($\sim 10^{-4}$ M) and upon excitation at 325 nm exhibits maximum fluorescence emission at 516 ± 2 nm (corrected) in buffered aqueous solution at pH 7.6 with a quantum yield of 0.21 and a lifetime of 11.8 ± 0.2 ns. The fluorescence of 3′-*O*-dansyladenosine is sensitive to the polarity of its solvent: in pyridine, a quantum yield of 0.61 at the emission maximum of 435 nm was observed. 3′-*O*-Dansyladenosine is a reversible competitive inhibitor of adenosine deaminase with a moderate inhibitive dissociation constant, $K_I = (1.54 \pm 0.13) \times 10^{-5}$ M. The enzyme–substrate analogue association constant was determined by equilibrium dialysis to be $K = (0.69 \pm 0.05) \times 10^5$ M^{-1}, very close to K_I^{-1}. The hydrophobic nature of its binding site in adenosine deaminase is evident from the strong blue shift of the fluorescence emission maximum to 440 nm, the 4-fold increase in fluorescence quantum yield, and the longer lifetime of 15.8 ± 0.2 ns; the tight, rigid nature of the complex is evident from its high fluorescence polarization value, 0.23. An 85% decrease in the fluorescence emission intensity of the adenosine deaminase–3′-*O*-dansyladenosine complex in the presence of adenosine indicates the selective binding to the enzyme active site. Correlation between the conformation of the probe, either when free in various solvents or when bound to the enzyme, and its fluorescence quantum yield is noted. 3′-*O*-Dansyladenosine is suitable for fluorescent labeling of adenosine deaminase in cell systems.

Adenosine has several important functions in cell metabolism. The first metabolic step of adenosine, e.g., in erythrocytes, is determined by two enzymes: adenosine deaminase (ADase)[1] and adenosine kinase (Parks & Agarwal, 1972). ADase plays an important role in the regulation of cellular growth and differentiation (Trotta & Bails, 1978). The absence of ADase activity in red cells is associated with a hereditary immunodeficiency ending in fatality (Giblett et al., 1972; Coleman et al., 1977). Also, ADase has the ability to catalyze the deamination of and thus inactivate several potent antitumor and antiviral nucleosides (Brink & Le Page, 1965; Schabel, 1968; Plunkett & Cohen, 1975). In addition, there is growing evidence of a not yet explained linkage between ADase specific activity and malignant transformation.

The correlation between the variable activities (Chiang et al., 1977; Trotta & Bails, 1978) of ADase due to malignant transformations and the corresponding biophysical and dynamical nature of this enzyme is being sought. It is proposed that the vertical position of this membranal enzyme (Agarwal & Parks, 1975; de Bruyn & Oei, 1974) being modulated by membrane modifiers (Nathan et al., 1979, 1980; Shinitzky & Souroujon, 1979; Shinitzky, 1976; Borochov & Shinitzky, 1976; Borochov et al., 1979; Shinitzky et al., 1979) may determine the activity of ADase through differential exposure of its active site to its natural substrate, adenosine.

We now report the synthesis, characterization, and spectroscopic nature of the first dansyl derivative of adenosine, 3′-*O*-dansyladenosine. It is a fluorescent competitive inhibitor of the deamination of adenosine by ADase and thus may be used as a fluorescent label of ADase, in the studies of the dynamics of ADase in malignant cells.

Experimental Procedures

Preparation of 3′-O-Dansyladenosine. A mixture of dansyl chloride (0.9214 g, 3.41 mmol) and adenosine (0.8717 g, 3.26 mmol) dissolved in 200 mL of dry pyridine was stirred at 4 °C in the dark for 15 h. Pyridine was evaporated in vacuo at a temperature below 50 °C, and 10 mL of cold water and 5 mL of chloroform were added to the gummy residue. Filtering and washing with 5 mL of acetone left a white amorphous residue, which was then washed with chloroform and again with acetone and finally dried in vacuo (10 mmHg, 25 °C); yield, 65%. Thin-layer chromatography, carried out on Eastman Chromatogram Sheets (6060 silica gel with fluorescent indicator, Kodak) and with a solvent system composed of ethanol–0.1 M ammonium acetate (40:1), exhibited a single spot at $R_f = 0.63 \pm 0.05$. Alternatively, TLC with 1-butanol–acetone–concentrated ammonia–water (50:40:3:15) exhibited a single spot at $R_f = 0.66 \pm 0.05$ (Kolassa et al., 1972).

Single crystals were grown from saturated ethanolic solution, into which acetone-containing capillaries were immersed. After a week in a sealed flask the crystals were collected.

Proton NMR was measured on a Bruker WH270 spectrometer (Weizmann Institute) in Me_2SO and Me_2SO-d_6 solutions (dried over 4-Å molecular sieves) and is reported in parts per million downfield from an internal standard of tetramethylsilane. The following peaks were observed: δ 2.74

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[1] Abbreviations used: ADase, adenosine deaminase; 3′-*O*-dansyladenosine, 3′-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl]adenosine; TLC, thin-layer chromatography; ϵ-adenosine, 1,N^6-ethenoadenosine; ϵ-ADP, 1,N^6-ethenoadenosine 5-diphosphate; ϵ-NAD, nicotinamide 1,N^6-ethanoadenine dinucleotide; GPDH, glyceraldehyde-3-phosphate dehydrogenase; CPK, Corey–Pauling–Koltun.

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(6 H, s) (dimethylamine); 3.08 (H, t) (5′-hydroxyl protons); 3.56–3.68 (2 H, m) (5′ protons); 4.00–4.04 (H, br d) (4′ proton); 4.20–4.24 (H, dd) (3′ proton); 4.64 (H, dd) (2′ proton); 5.96 (H, d) (1′ proton); 7.16 (2 H, s) (NH_2 protons); 7.46–8.60 [br m containing the remaining protons, in particular 8.20 (H, s) and at 8.43, identified as the 2 and 8 protons].

Mass spectra, recorded on an Atlas CH-4 MS mass spectrometer (Weizmann Institute) at 70 eV (source temperature 170 °C; probe temperature 75 °C), did not show the molecular ion. However, the fragmentation peaks reported for both adenosine (Biemann & McCloskey, 1962) and the dansyl group (Seiler et al., 1970; Addeo et al., 1974), e.g., *m/e* 135 (100%) $[C_5H_5N_5]^+$, 136 (100%) $[C_5H_6N_5]^+$, and 170 (15.6%) dimethylaminonaphthalene ion, were observed. An additional peak, *m/e* 250 (51%), which may be attributed to the dimethylaminnaphthalenesulfonyl ion, is indicative of the linkage between the dansyl and adenosine moieties in the product. The infrared spectrum (on a Perkin-Elmer Grating IR Spectrometer 377) of a KBr pellet showed strong peaks at 1215, 1105, and 1080 cm^{-1}, characteristic of aromatic O–SO_2 stretching modes. Anal. Calcd for $C_{22}H_{24}N_6O_6S \cdot 2.5H_2O$; C, 48,44; H, 5.32; S, 5.85; N, 15.43. Found: C, 48.17; H, 5.56; S, 6.11; N, 15.31.

The periodate–benzidine test for glycols (Viscontini et al., 1955) gave negative results, indicating that either the 2′ or the 3′ site was bound to dansyl. This agrees with the NMR indication of an unbound 5′ site.

The presence of exclusively 3′-*O*-dansyladenosine rather than 2′-*O*-dansyladenosine or a mixture of the two isomers was proven by using the reaction due to Robins et al. (1974). It was the monomethylation of nucleosides by diazomethane, using stannous chloride as a catalyst. To a suspension of our compound in 1,2-dimethoxyethane was added a methanolic solution of 10^{-3} M $SnCl_2 \cdot 2H_2O$ and an ethereal solution of diazomethane, and the suspension was stirred overnight. The system was dried by evaporation, and hydrolysis was performed in a 10% bicarbonate solution. The solution was applied to a column of Dowex 1-X2, 50–100 mesh, and developed in an EtOH–H_2O (2:1) solvent; 10-mL fractions were collected and monitored by TLC. NMR of the eluent showed all the peaks characteristic of dansyladenosine plus a new peak at δ 3.49, characteristic of the 2′-*O*-methyl derivative (mp after recrystallization from ethanol, 202–203 °C). In conclusion, only 2′ could be methylated, because 3′ was protected by dansyl binding.

Spectroscopic Determinations. Absorption spectra were recorded at 25 °C on Cary 17 and Bausch & Lomb Spectronic 210 UV. Molar absorption coefficients were determined from plots of the measured absorbance at the desired wavelengths vs. different concentrations of 3′-*O*-dansyladenosine in 63 mM buffer phosphate, pH 7.6 (reference solution). Molar absorption coefficients of 3′-*O*-dansyladenosine in pyridine (anal., Merck) and in *N*,*N*′-dimethylformamide (anal., Frutarom) were similarly determined.

Steady-state fluorescence studies were carried out on a Perkin-Elmer MPF-44 spectrofluorometer equipped with polarizers and a thermostated cell holder. A RTE-3 (Neslab Instrument Co.) thermostatic bath was used for control of temperature, which was held at 37 ± 0.5 °C. Corrected spectra were obtained with a differential corrected spectra unit 063-0186 by using Rhodamine B (anal., Research Organic Inc.) for calibration.

Quantum yield of 3′-*O*-dansyladenosine was determined by comparing its relative fluorescence yield (excitation at 325 nm) with that of quinine sulfate in 1 N H_2SO_4, assumed to have an absolute quantum yield at 0.70 (Scott et al., 1970). Polarization excitation spectra were calculated (Fuchs et al., 1975; Parola et al., 1979) point by point by varying the wavelength of excitation and following the fluorescence emission at its maxima.

Fluorescence Lifetime Studies. Fluorescence decay curves were obtained with a nanosecond single photon counting spectrofluorometer (Parola et al., 1979; Chen et al., 1977; Kawato et al., 1977; Hildenbrand & Nicholau, 1979; Veatch & Stryer, 1977). The sample was excited at the 355-nm nitrogen emission line generated by a free running flash lamp. The sample holder was thermostated at 37 °C. The emission at a right angle, filtered by a band-pass filter (Baird-Atomic) which transmits light at 419 nm (band width 10 nm), was detected by an RCA 8850 photomultiplier. An Ortec time to pulse-height converter and an Elscint-Meda multichannel analyzer were used to obtain the fluorescence decay data. The lamp profile was obtained prior to and after the sample measurements. Analysis for a convoluted sum of exponential decay terms was done by the nonlinear least-squares method, employing first-guess parameters obtained by the method of moments (Chen et al., 1977).

ADase Kinetic Studies. The general assay procedure was based on either following the rate of disappearance of the absorption band of adenosine at 265 nm (Kaplan, 1955) or detecting the ammonia liberated due to adenosine deamination. Ammonia was determined by slightly modifying the methods described by Seligson & Seligson (1951), Keller et al. (1967), and by Rogler-Brown et al. (1978), based on the Berthelot reaction.

3′-*O*-Dansyladenosine was assayed as a substrate for ADase. The result was negative. A trace of absorbance at 625 nm (0.03) was the result of an interaction with phenol added in the ammonia assay and was not caused by the enzyme.

Kinetic Inhibition Test. A series of assay tubes were closed with septum rubbers, each containing 1 mL of 6.06×10^{-10} M ADase (Sigma Type II, activity 0.95 unit/mg of protein) with variable volumes of a stock solution containing 9.45×10^{-4} M adenosine (Sigma). A total volume of 9 mL was prepared. Three more series of assay tubes, each containing, in addition to the above, 1 mL of 0.42, 1.20, and 2.40×10^{-4} M, respectively, 3′-*O*-dansyladenosine (total volume 9 mL), were similarly prepared. Two additional blank tubes, one containing only the enzyme and the other containing the enzyme and 3′-*O*-dansyladenosine, were also prepared. All tubes were incubated for 1 h at 36 °C. The enzymatic reaction was then terminated by addition with a syringe of 1 mL of 0.606 M trichloroacetic acid and 0.325 M NaOH in distilled water. This was followed by the addition of 1 mL of a water solution of 1 mM sodium prusside and 0.53 M phenol and 1 mL of 0.03 M sodium hypochlorite (in 0.63 M NaOH). The absorbance of the resultant dyestuff at 623 was determined against an aqueous phosphate buffer, pH 7.6, as a blank. Solutions of ammonium sulfate were used to calibrate the rate of liberation of ammonia. The same samples were used when the absorbance at 265 nm was followed.

Results

Absorption Spectrum of 3′-O-Dansyladenosine. 3′-*O*-Dansyladenosine (decomposition at 165–169 °C) is partially soluble in water ($\sim 10^{-4}$ M). Its UV spectrum in phosphate buffer, pH 7.6 (Figure 1), exhibits three principal peaks at 213–214 nm ($\epsilon = 59.2 \times 10^3$), at 240–245 nm ($\epsilon = 24.4 \times 10^3$), and at 318–330 nm ($\epsilon = 6.47 \times 10^3$). The observed UV absorption is a superposition of the individual contributions of the broad dansyl absorption between 305 and 320 nm which

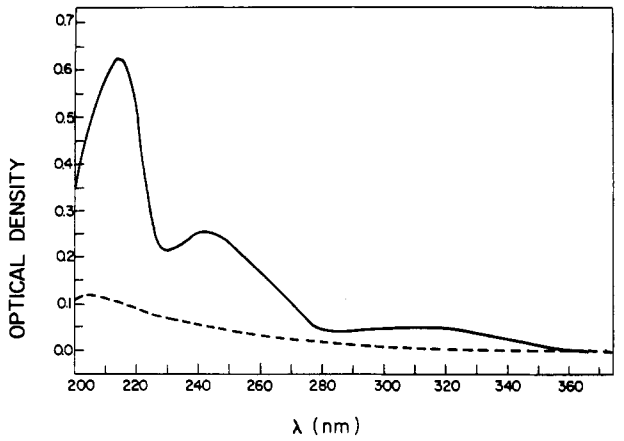

FIGURE 1: Absorption spectrum of 3′-*O*-dansyladenosine (—) in 63 mM phosphate buffer, pH 7.6. Solvent base line is shown too (---).

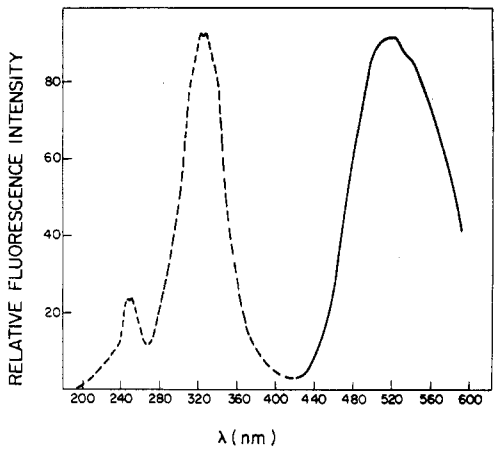

FIGURE 2: Fluorescence excitation (---) (noncorrected) and emission (—) (corrected) spectra of 3′-*O*-dansyladenosine in 63 mM phosphate buffer, pH 7.6.

is composed of at least two different electronic transitions (Stryer, 1965; Hudson & Weber, 1973) and the π–π^* transitions of both the dansyl (λ_{max} at 215 and 238 nm) and the adenosine (λ_{max} at 208 and 257 nm) moieties (Clark & Tinoco, 1965).

Fluorescence Properties of 3′-O-Dansyladenosine. With excitation at λ_{max} = 325 nm, the corrected fluorescence emission spectrum of 3′-*O*-dansyladenosine in phosphate buffer, pH 7.6 (Figure 2), exhibited maximum emission at λ = 514–518 nm. The fluorescence quantum yield of 3′-*O*-dansyladenosine, Φ_{325} = 0.21, is lower than that of dansylic acid (Himel & Mayer, 1970), yet is still quite substantial for an aqueous medium.

Figure 3 shows the noncorrected fluorescence emission spectra of 3′-*O*-dansyladenosine in three of the solvents in which it is soluble, i.e., pyridine, dimethylformamide, and water (phosphate buffer, pH 7.6). 3′-*O*-Dansyladenosine is shown to retain the sensitivity of dansyl chloride to solvent polarity, as measured by its fluorescence emission maximum and quantum yield. Thus Φ_{325} = 0.61 in pyridine at the blue-shifted fluorescence emission maximum (435 nm) is almost 3 times higher than that in water, which is more polar.

The fluorescence emission of 3′-*O*-dansyladenosine in phosphate buffer, pH 7.6, exhibited a single exponential decay with a lifetime of 11.8 ± 0.2 ns (Figure 4).

3′-O-Dansyladenosine Binding to ADase. The binding constant was determined from equilibrium dialysis experiments carried out in a Cellofan Tubing celluloid sleeve (Cenco, 70160-1) containing a fixed quantity of the enzyme. A series

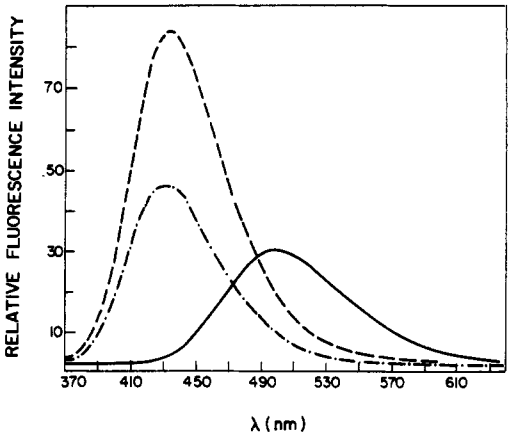

FIGURE 3: Fluorescence emission spectra of 3′-*O*-dansyladenosine in 63 mM phosphate buffer, pH 7.6 (—), *N*,*N*′-dimethylformamide (-·-), and pyridine (---). Excitation was at 325 nm. Equal concentrations of 3′-*O*-dansyladenosine, 3.30 × 10^{-5} M, were used in all solvents.

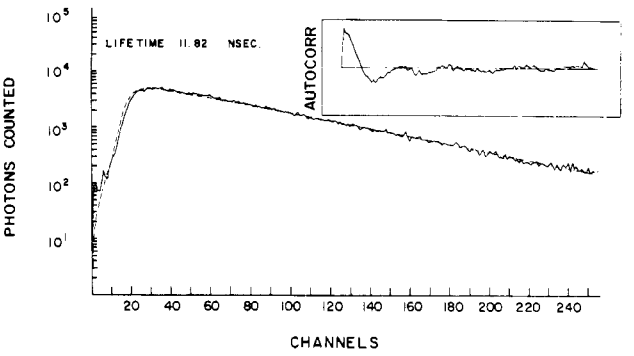

FIGURE 4: Fluorescence emission decay of 3′-*O*-dansyladenosine (3 × 10^{-5} M) in phosphate buffer at 37 °C (—). Convoluted single-exponential decay, calculated for τ = 11.82 ns, is shown too (---). The width of the lamp flash was 2 ns. The autocorrelation function of the residuals (upper right corner) is shown; timing calibration was 0.21 ns/channel.

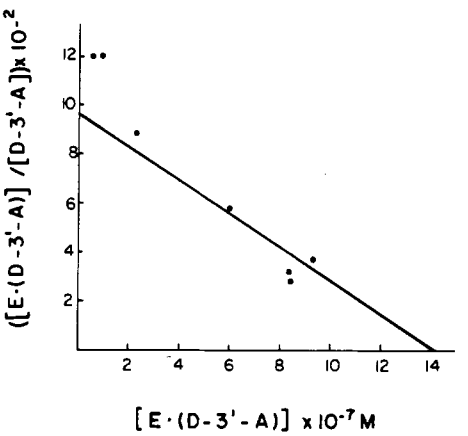

FIGURE 5: Scatchard plot of ADase–3′-*O*-dansyladenosine binding. The ADase concentration was 5.85 g/L (0.71 × 10^{-6} M) in phosphate buffer. The association constant of (0.69 ± 0.05) × 10^5 M^{-1} was derived from the slope.

of experiments, in which the concentration of the substrate analgoue was varied, was run at 4 °C. Each run lasted 18 h. Samples of the solution outside the sleeve were assayed fluorometrically at 37 °C, λ_{ex} = 325 nm and λ_f = 505 nm. The data are presented in a Scatchard plot (Figure 5). The slope yielded the association constant K = (0.69 ± 0.05) × 10^5 M^{-1}.

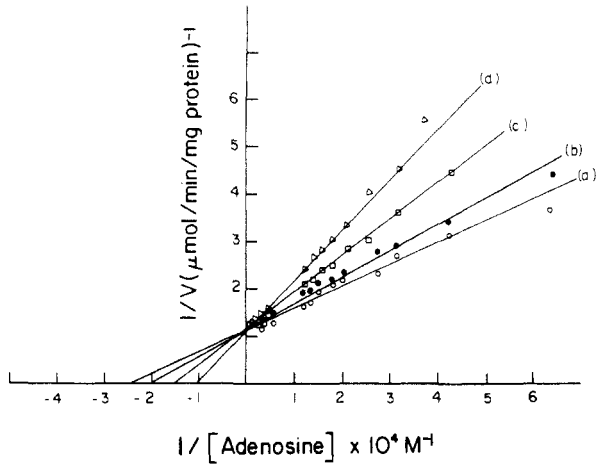

FIGURE 6: Lineweaver–Burk plot for inhibition of ADase by 3′-*O*-dansyladenosine. The activity of ADase was measured as described under Experimental Procedures. Straight lines were determined by the least-squares method. The ADase concentration was 6.06×10^{-10} M in phosphate buffer. 3′-*O*-Dansyladenosine concentrations: (a) 0; (b) 0.42×10^{-4} M; (c) 1.20×10^{-4} M; (d) 2.4×10^{-4} M. Competitive inhibition is indicated. $K_I = (1.54 + 0.13) \times 10^{-5}$ M.

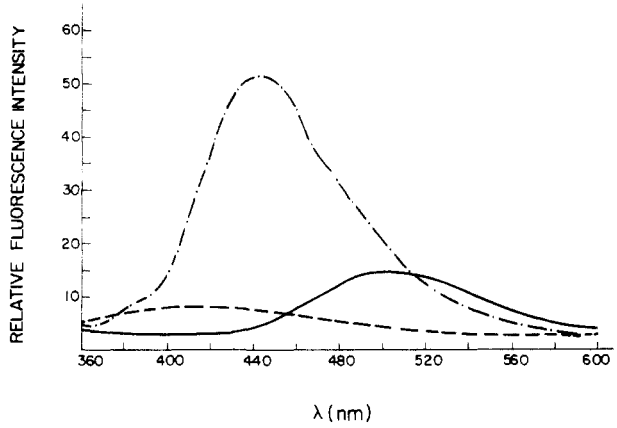

FIGURE 7: Noncorrected fluorescence emission spectra of a phosphate buffer pH 7.6 solution of 3′-*O*-dansyladenosine (3.30×10^{-6} M, λ_{ex}^{max} = 325 nm) (—), or 3′-*O*-dansyladenosine bound to ADase (1.64×10^{-6} M, λ_{ex}^{max} = 340 mn) (-·-), and of ADase (1.64×10^{-6} M, λ_{ex} = 340 nm) in the absence of substrate (---).

Enzymatic Studies of 3′-O-Dansyladenosine. As stated above, 3′-*O*-dansyladenosine is not a substrate for ADase.

Inhibition Test. Figure 6 shows the Lineweaver–Burk plot for the deamination of adenosine by ADase in both the presence and the absence of 3′-*O*-dansyladenosine. The Michaelis contant for adenosine, $K_M = 4.07 \times 10^{-5}$ M, obtained by least-squares treatment, is similar to that obtained by following the 265-nm absorbance of adenosine, $K_M = (4.07 \pm 0.08) \times 10^{-5}$ M, and to those reported in the literature, e.g., $K_M = 4 \times 10^{-5}$ M (Ronca & Zucchelli, 1968) or $K_M = 4.2 \times 10^{-5}$ M (Le Page & Junga, 1965). By following the rate of liberation of ammonia, it is possible to eliminate complications which could arise from the absorbance by the dansyl moiety close to 265 nm, at which wavelength the disappearance of adenosine could alternatively be followed. A background color reaction on the order of <5% originating in the phenol added during the ammonium assay, was subtracted from the activity observed for adenosine when measured in the presence of 3′-*O*-dansyladenosine (see Experimental Procedures). 3′-*O*-Dansyladenosine is shown in Figure 6 to be a reversible competitive inhibitor for the deamination of adenosine with an inhibitive dissociation constant, $K_I = (1.58 \pm 0.12) \times 10^{-5}$. The near agreement of this K_I with the reciprocal of the association constant K indicates that adenosine and its fluorescent substrate analogue compete for the same binding site on the enzyme.

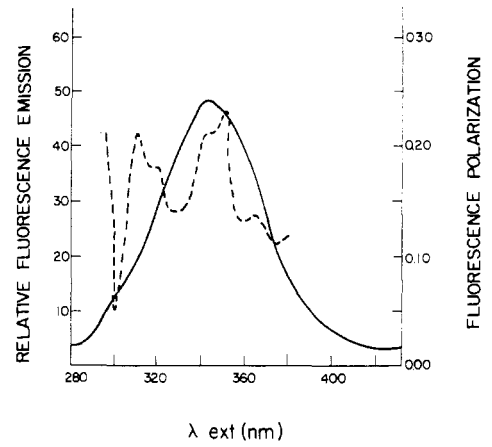

FIGURE 8: Noncorrected fluorescence excitation spectrum (—) and excitation polarization spectrum (---) of a phosphate buffer pH 7.6 solution of 3′-*O*-dansyladenosine (3.30×10^{-6} M) bound to ADase (1.64×10^{-6} M). The fluorescence excitation spectrum was recorded at 440-nm emission maximum. The excitation polarization spectrum, followed at the fluorescence emission maximum, was carried out in duplicate (average values are presented) and corrected for a blank containing the enzyme solution only.

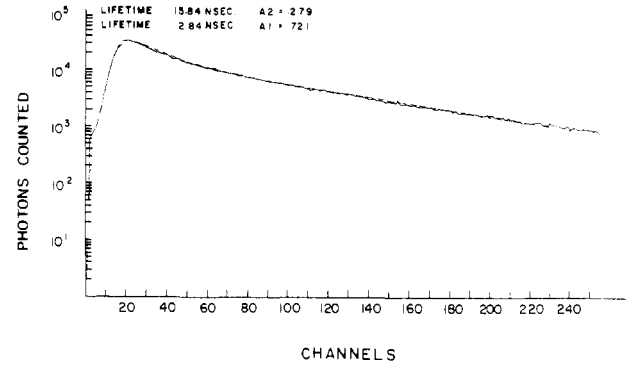

FIGURE 9: Fluorescence emission decay of 3′-*O*-dansyladenosine (3.30×10^{-6} M) bound to ADase (1.64×10^{-6} M) in phosphate buffer at 37 °C (—). Convoluted double-exponential decay calculated for τ_1 = 15.84 ns and τ_2 = 2.84 ns is shown. The half-height width of the lamp flash was 2 ns. Timing calibration was 0.21 ns/channel.

Fluorescence Properties of the ADase–3′-O-Dansyladenosine Complex. Figure 7 compares the fluorescence emission spectra of the free and the bound 3′-*O*-dansyladenosine. Residual enzyme fluorescence is shown too. A blue shift in the fluorescence emission maximum from 500–510 to 440 nm, a red shift in the fluorescence excitation maximum from 325 to 340 nm, and an almost 4-fold increase in maximum fluorescence emission intensity take place upon complexation of 3′-*O*-dansyladenosine; $\Phi_{325nm} = 0.75$. Figure 8 exhibits both the excitation polarization spectrum and the fluorescence excitation spectrum of the ADase–3′-*O*-dansyladenosine complex. The latter is indicative of at least two emitting excited states, as was already noted before for other dansyl derivatives (Hudson & Weber, 1973). The maximum fluorescence polarization value obtained for the bound 3′-*O*-dansyladenosine is $P = 0.23$ at $\lambda_{ex} = 350$ nm and $\lambda_{em} = 440$ nm. The free 3′-*O*-dansyladenosine, in the same buffer solution, exhibited complete fluorescence depolarization, with $P = 0.00$.

The fluorescence emission of the ADase–3′-*O*-dansyladenosine in phosphate buffer, pH 7.6, as a function of the time (Figure 9), exhibited a double-exponential decay. The

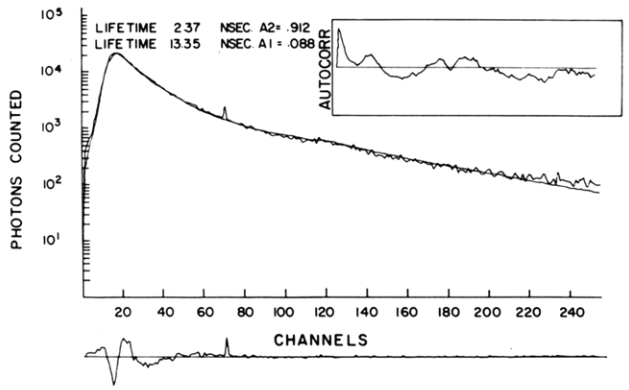

FIGURE 10: Fluorescence emission decay of ADase (1.64×10^{-6} M) in phosphate buffer at 37 °C. Convoluted double-exponential decay calculated for predominantly $\tau_1 = 2.37$ ns is shown. Other details are indicated in Figure 4 and under Experimental Procedures.

shorter lifetime, $\tau = 2.8$ ns, may be attributed to the lifetime of the enzyme blank alone or may originate from some impurities in it. An attempt to analyze the emission decay course of the enzyme blank by a single-exponential function revealed a similar lifetime of ~2.4 ns (Figure 10). The longer lifetime, $\tau = 15.8 \pm 0.2$ ns (Figure 9), is ascribed to the ADase–3′-*O*-dansyladenosine and is indicative of its hydrophobic environment in the ADase site.

Upon addition of adenosine (0.01 M) to the ADase–3′-*O*-dansyladenosine complex (ADase 4.66×10^{-5} M; 3′-*O*-dansyladenosine 9.4×10^{-7} M), an 85% decrease in fluorescence intensity at $\lambda_{max} = 440$ nm was observed. The decrease in fluorescence intensity of the complexed 3′-*O*-dansyladenosine is not due to direct quenching by adenosine, since the fluorescence emission intensity of the free 3′-*O*-dansyladenosine was not altered in the presence of saturated adenosine solutions. The observed decline in the fluorescence intensity is compatible with the reversibly competitive nature of our substrate analogue.

Discussion

ϵ-Adenosine analogues, initially introduced by Leonard (Secrist et al., 1972), have been successfully utilized in the study of adenylate kinase, hexokinase, phosphofructokinase, and pyruvate kinase. While highly fluorescent, with a quantum yield close to 0.6, ϵ-adenosine presents a modification of adenosine at the 6-NH_2 group, which is the reaction site of ADase, and thus may not be a suitable fluorescent analogue for the study of this enzyme. ϵ-Adenosine analogues exhibit little or no sensitivity to the polarity of the media and thus may not probe the microenvironmental changes essential for biological studies. Furthermore, though ϵ-adenosine analogues have considerable Stokes shifts, $\lambda_{ex}^{max} = 303$ nm and $\lambda_{em}^{max} = 415$ nm (at pH 7.0), it would be highly desirable to employ fluorescent probes with even larger Stokes shifts, particularly for use in labeling scattering cell systems. We have thus chosen to attach the relatively bulky dansyl group to adenosine, at a site remote from the reactive center of this substrate with ADase, i.e., at the 3′ site of the ribose moiety (Baker, 1967).

Dansyl chloride is known to react with primary and secondary amino groups as well as with hydroxyl groups (Gray, 1967). Dansyl derivatives strongly fluoresce and can be detected in minute quantities, with sensitivity comparable to analytical radioactive isotope methods (Seiler, 1970). Furthermore, dansyl derivatives exhibit a strong dependence of the fluorescence characteristics on solvent dielectric constant (Chen, 1967). Yet, to our surporise, the reported syntheses of dansyladenosine are most rudimentary, and the product

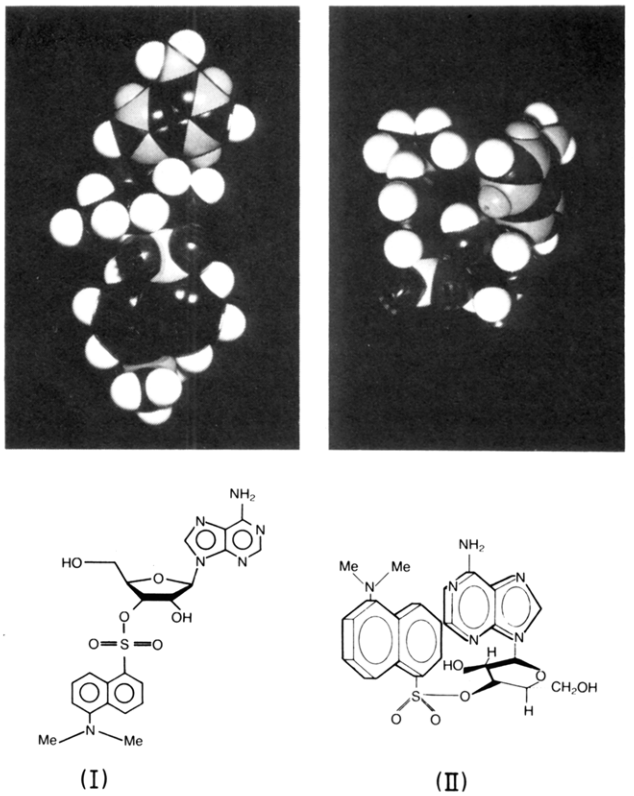

FIGURE 11: Structural models of 3′-*O*-dansyladenosine: (I) open conformation; (II) sandwich conformation.

analysis is either lacking or very qualitative (Wada et al., 1977; Nakamura et al., 1977; Osborne & Neuhoff, 1977), the major emphasis being rather on development of sensitive TLC detection methods for dansyladenosine in biological tissues.

In an attempt to establish the biophysical characteristics of ADase in normal and malignant cells (Parola et al., 1979; Parola & Souroujon, 1979; Rosenthal et al., 1978; Fuchs et al., 1975), it is desirable to fluorescently label this enzyme selectively with a chemically well-defined label. To this end, we initially undertook the first careful synthesis and characterization of the 3′-*O*-dansyladenosine (structure I of Figure 11).

The reaction was performed under mild conditions, leading to no binding of dansyl chlroide to either the 6-NH_2 or the 5′-OH on adenosine, as evident from the 270-MHz proton NMR obtained in Me_2SO, dried with molecular sieves. The lack of reactivity of the 6-NH_2 moeity could arise from intramolecular hydrogen bonding, known to occur in various amino heterocycles (Albert, 1966). The 2′- and 3′-hydroxyl protons, to be expected at δ 5.65 and 5.40 (Townsend, 1973), were not observed. Instead, an additional hydrogen was observed in the aromatic region, δ 7.56–7.60, which disappears in the presence of trace amount of water, indicating its hydroxylic origin. We thus conclude that the 1:1 adduct (elemental analysis) between adenosine and dansyl chloride was formed either at the 2′- or 3′-hydroxyl moiety. Internal hydrogen bonding between, say, the 2′-OH and the sulfoxy oxygen (if binding occurred, say, at the 3′-OH) is an unlikely explanation for the shift to the aromatic region since it requires the formation of an unstable seven-membered ring. CPK space-filling models of the two possible 1:1 adducts between adenosine and dansyl chloride reveal that the 3′-*O*-dansyladenosine could be stabilized in an aqueous solution by assuming a sandwichlike structure (structure II of Figure 11) in which the aromatic rings overlap, being bridged by the ribose moiety. This conformation is preferred over the ex-

Table I: Fluorescence Characteristics of Free and Bound 3′-*O*-Dansyladenosine[a]

3′-*O*-dansyladenosine	$\lambda_{ex,nm}^{max}$ [b]	$\lambda_{em,nm}^{max}$ [b]	Φ[d]	τ_1 (ns)	p[d]
free	325	505 (516)[c]	0.21	11.8 ± 0.2[f]	0.00
bound	340	440	~0.8[e]	15.8 ± 0.2[g]	0.21

[a] Experimental details are given in Experimental Procedures. [b] Obtained from noncorrected fluorescence spectra. [c] Corrected fluorescence spectrum. [d] At maximum excitation and emission wavelength. [e] Based on the ratio in fluorescence emission intensities between the free and bound probe. [f] Obtained from single-exponential decay analysis. [g] Obtained from double-exponential decay analysis.

tended open conformation, since it allows hydrophobic interactions between the aromatic rings and also hydrophilic interactions of the still exposed ribose moiety. This conformation renders this molecule at least partially soluble (up to 10^{-4} M) in aqueous solution, despite its high molecular weight and its aromatic nature. The solubility of the fluorescent substrate analogue is obviously a prerequisite for its application to cell systems in buffered aqueous solutions. 2′-*O*-Dansyladenosine, however, would preferentially assume an extended open conformation, which would require higher solvation energies in water. Most striking, though, is the observation that in 3′-*O*-dansyladenosine in the sandwich conformation, the 2′-OH is posed between the two aromatic rings, sidewise to the aromatic hydrogens. This orientation exposes the 2′-OH hydrogen to aromatic ring currents which could account for the aforementioned chemical shift into the aromatic region. No such ring currents could possibly affect the 3′-OH of 2′-*O*-dansyladenosine; CPK space-filling models of this compound reveal that the 3′-OH assumes a rather extended orientation, away from the hydrophobic aromatic region into the aqueous environment. This modeling strengthens our conclusion, based on chemistry (vide supra), that 3′-*O*-dansyladenosine is the product obtained. Conclusive structural evidence can be obtained from X-ray crystal structure analysis, which will be undertaken. It is noteworthy that the sandwich conformation of 3′-*O*-dansyladenosine leaves one face of the adenosine substrate essentially unaffected by the presence of the bulky dansyl group. This would suggest an effective binding of this substrate analogue to the enzyme in spite of burdening the adenosine by the relatively bulky dansyl moiety (see below).

The UV spectrum of 3′-*O*-dansyladenosine (Figure 1) does not indicate any new absorption bands which could be expected if specific (e.g., charge-transfer) interactions between the two aromatic rings (Parola & Cohen, 1980) had taken place; solvation and desolvation interactions may thus account for the proposed conformation. The reduced fluorescence quantum yield of 3′-*O*-dansyladenosine, relative to dansylic acid (~50%), could presumably result from quenching effects by the overlapping adenine group. Interestingly, 6-dansyladenosine was found to be essentially nonfluorescent in aqueous solution (G. Skorka and A. H. Parola, unpublished results). In the latter case, CPK space-filling models indicate an even tighter packing, possibly leading to stronger quenching interactions of the two aromatic moieties. No such tight packing is possible in 3′-*O*-dansyladenosine, due to the intervening 2′-OH group. Thus, the observed trend of reduction in fluorescence quantum yield, down the series of dansyl-OH, 3′-*O*-dansyladenosine, and 6-dansyladenosine, correlates well with the potential interactions between the adenine and naphthalene moieties possibly leading to physical quenching. For the latter two compounds, fluorescence quantum yields increase in the less polar pyridine, which may, at least partially, induce a more extended conformation of the solute. The binding of these probes to the hydrophobic active site of ADase would thus lead to a conformational change in these probes, leading to an increased quantum yield of fluorescence of the enzyme-bound probe. Such conformational changes were recently reported by Gafni (1977) for the binding of ε-NAD to GPDH, as studied by fluorescence decay kinetics and by X-ray crystallography.

The fluorescence spectral characteristics of 3′-*O*-dansyladenosine (Figure 2) make it suitable for labeling cell systems: the λ_{ext}^{max} = 325 nm is removed from the protein excitation maxima as compared with ε-adenosine (λ_{ext}^{max} = 303 nm), and the very large Stokes shift of ~190 nm (as compared with 112 nm in ε-adenosine) makes it more suitable for studying scattering cell systems.

3′-*O*-Dansyladenosine is a good polarity probe (Figure 3), exhibiting a blue shift of ~80 nm and a 3-fold increase in the fluorescence quantum yield in the less polar pyridine as compared to aqueous solution. The single-exponential decay of the fluorescence of 3′-*O*-dansyladenosine (Figure 4) is somewhat surprising in view of the mixed electronic states (at least two) indicated by the absorption spectrum (Figure 1) and by the fluorescence excitation polarization spectra reported for other dansyl derivatives (Hudson & Weber, 1973).

3′-*O*-Dansyladenosine is a reversible competitive inhibitor of ADase (Figure 6), with a moderate inhibition constant. The very similar binding affinity to ADase of adenosine and of 3′-*O*-dansyladenosine may be accounted for by the proposed sandwich conformation. This conformation renders one face of the molecule unaltered, with the dansyl group hooked behind.

That the specific binding of 3′-*O*-dansyladenosine occurs at the active site of ADase gains support from the almost complete (85%) reduction in the fluorescence emission intensity of the ADase–3′-*O*-dansyladenosine complex upon addition of free adenosine. The hydrophobic nature of the binding is spectroscopically evident by the strong blue shift of the fluorescence emission maximum (Figure 7), by the 3–4-fold increase in fluorescence intensity, and by the increase in fluorescence lifetime from 11.8 ± 0.2 ns for the free probe to 15.8 ± 0.2 ns for the enzyme-bound probe.

The rigid nature of the ADase–3′-*O*-dansyladenosine complex may be inferred from its remarkably high fluorescence polarization value, P = 0.21–0.23 (Figure 8), as compared with the free probe (P = 0.0). In other systems reported in the literature, e.g., the pyruvate kinase–ε-ADP complexes, reported P values were rather small, i.e., in the range of 0.005 (Barrio et al., 1973).

In conclusion, 3′-*O*-dansyladenosine is a reversibly competitive, fluorescent substrate analogue for ADase. It binds to the hydrophobic active site of ADase as effectively as adenosine, to form a rigid complex. It exhibits high sensitivity to the polarity of its environment and is spectroscopically distinguishable (Table I) when free or bound to the enzyme. Since the ADase–3′-*O*-dansyladenosine complex exhibits a large Stokes shift and a high fluorescence quantum yield, up to ~0.8, this label would be suitable for studying turbid labeled cell suspensions even with low ADase content. Its fluorescence lifetime of ~16 ns renders it suitable for dynamical studies (rotational relaxation times) of this loosely bound membrane enzyme. Finally, 3′-*O*-dansyladenosine could be converted into a photoaffinity label such as 8-azido-3′-*O*-dansyladenosine (G.

Skorka and A. H. Parola, unpublished results) which, after reversible binding in the dark, could be photochemically converted into the reactive, covalently binding nitrene (Dreyfus et al., 1978). These studies will be published elsewhere.

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