

Equilibrium studies of a fluorescent tacrolimus binding to surfactant protein A [☆]

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Abstract

Tacrolimus (FK506) is a hydrophobic immunosuppressive agent used in kidney, liver, and lung transplantation. The objective of this study was to characterize the binding of FK506 to surfactant protein A (SP-A), an abundant lipoprotein found in the alveolar fluid that functions as part of the innate immune system in the lung. We have synthesized a novel derivative of FK506 in which a dansyl moiety was covalently bound via cadaverine to the C22 position of the FK506 molecule (DNS-FK). Using the fluorescence and anisotropy properties of DNS-FK, we demonstrated that tacrolimus avidly binds to SP-A with an apparent equilibrium association constant (K_{app}) of 10^7 M^{-1} and a Gibbs binding free energy of $-40 \text{ kJ mol}^{-1} \text{ K}^{-1}$. Derivatization of FK506 at the C22 position did not block FK506 binding to the cytosolic immunophilin FK506-binding protein (FK-BP) or human serum albumin (HSA), both used as controls of tacrolimus-binding proteins. K_{app} for FK-BP/DNS-FK and HSA/DNS-FK complexes were 1.5×10^7 and 10^7 M^{-1} , respectively. The high sensitivity of this analytical technique makes it suitable for binding analysis of FK506 to proteins. © 2005 Elsevier Inc. All rights reserved.

Keywords: Fluorescent tacrolimus; Dansylcadaverine-FK506; SP-A; FK506 binding protein; Human serum albumin; Fluorescence anisotropy assay

Tacrolimus, also known as FK506 (Fig. 1), is a hydrophobic macrolide lactone produced by *Streptomyces tsukubaensis* [1] that acts as a powerful and clinically useful immunosuppressant through disruption of signaling events mediated by calcineurin in T lymphocytes [2]. Clinical trials have shown tacrolimus to be an effective alternative to cyclosporin for both primary immunosuppression after solid-organ transplantation and rescue therapy for acute rejection recipients [3].

Local immunosuppression is a potential approach to reduce the adverse effects of systemic immunosuppression. Inhaled tacrolimus administration after lung transplantation could be an alternative to its systemic or oral administration. Using this route, tacrolimus would reach

the alveolar fluid, where it would interact with alveolar macrophages, lymphocytes, and epithelial cells in a fluid environment characterized by the presence of pulmonary surfactant. The latter is a macromolecular complex composed of 90% lipids and 10% proteins that create a lipid-rich phase separating alveolar gas and liquid at the surfaces of alveolar epithelial cells [4,5]. Surfactant protein A (SP-A)¹ is the most abundant pulmonary surfactant protein. SP-A is a large oligomeric protein of approximately 650 kDa, composed of 18 nearly identical subunits. Each SP-A subunit contains an amino-terminal collagen-like domain and a carboxyl-terminal globular

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¹ Abbreviations used: DNS, dansylcadaverine; DNS-FK, dansylcadaverine-FK506; FK506, tacrolimus; FK-BP, FK506-binding protein; HSA, human serum albumin; SP-A, surfactant protein A; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; FRET, fluorescence resonance energy transfer; ESI, electrospray ionization.

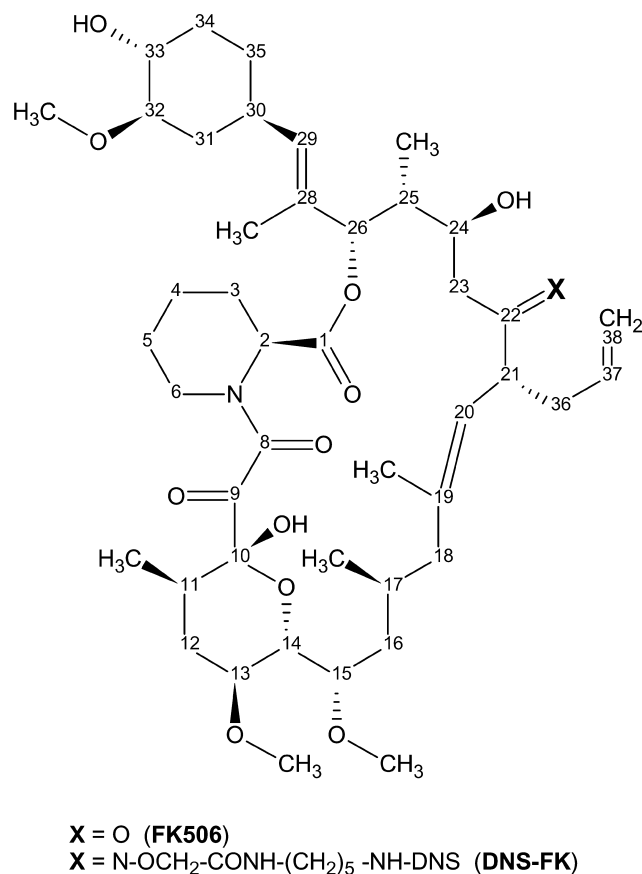


Fig. 1. Chemical structure of tacrolimus (FK506) and the novel fluorescent derivative (DNS-FK).

domain (involved in phospholipid and carbohydrate binding) that are linked by a more hydrophobic domain (neck). Electron microscopy show that recombinant human SP-A and native SP-A assemble as six trimers arrayed in parallel and in register, resembling a bouquet of tulips [6,7].

SP-A is involved in innate immune host defense and inflammatory immunomodulator processes of the lung [8,9]. Experiments with mice deficient in SP-A demonstrate that SP-A knockout mice exhibit increased susceptibility to bacterial and viral infections and enhanced inflammatory responses in the lung to a variety of stimuli [10]. Nearly all alveolar SP-A is complexed with surfactant phospholipids. Given the capability of SP-A to interact with a broad range of lipids [11], it is conceivable that SP-A binds to hydrophobic drugs such as FK506. This hypothesis is supported by the finding that the lipid aggregation activity of SP-A, which is of relevance in pulmonary surfactant biology, is markedly increased in the presence of FK506 [12]. The potential binding of FK506 to SP-A might influence its bioavailability in the alveolar fluid and affect drug absorption or FK506 immunosuppressive activity. At the same time, binding of FK506 to SP-A might affect SP-A's functions.

Most of the studies on drug–protein interaction are performed using techniques that require nonphysiologi-

cal conditions such as high protein concentrations, deuterated solvents, or crystallized proteins. Our approach to investigating tacrolimus binding to proteins is to prepare a derivative or analogue in which a fluorescent probe is chemically attached to the molecule by covalent bonding as described for other molecules with possible or recognized pharmacological activity [13–16].

The use of substituted aminonaphthalenesulfonates as fluorescent probes in proteins and other macromolecules is well known [17–20]. These compounds show characteristic changes in the location of their fluorescence maxima and emission intensities as a function of solvent polarity. In addition, changes in the fluorescence polarization of these moieties make it possible to study the interaction of small molecules with proteins.

This work describes for the first time the synthesis of a derivative of FK506, namely dansyl-FK506, bearing a fluorescent tag in a position that does not block its interaction with proteins that are known to bind to FK506, such as the cytosolic immunophilin FK506-binding protein (FK-BP) [21–23] and serum albumin [24]. The only fluorescent tacrolimus reported so far has been its dansyl hydrazide generated in situ for HPLC detection of the drug [25]. Using dansyl-FK506, we show here for the first time that FK506 binds to SP-A with an apparent equilibrium association constant of 10^7 M^{-1} , considering a molecular mass of 36 kDa for the monomer of human SP-A, and a Gibbs binding free energy of ca. $-40 \text{ kJ mol}^{-1} \text{ K}^{-1}$.

Materials and methods

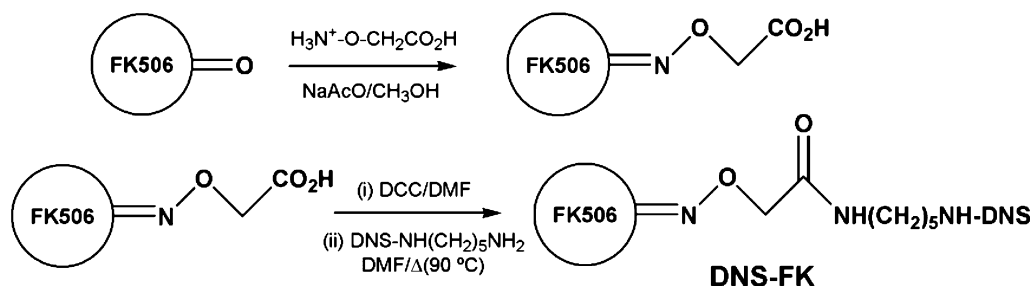
Materials

FK506 was provided by Fujisawa GmbH (Munich, Germany). A spectroscopic characterization of this material has been published [26]. Dansylcadaverine was purchased from Molecular Probes (Eugene, OR). FK506-binding protein and fatty-acid-free human serum albumin (HSA) were obtained from Sigma–Aldrich (St. Louis, MO) and used without further purification. Carboxymethoxylamine hemichlorhydrate and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich (Madrid, Spain). Chloroform and methanol were of spectroscopy grade, purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade and obtained also from Merck.

Synthesis of DNS-FK506

Synthesis of carboxymethoxyloxime of FK506 at C22

The FK506 derivatization procedure to obtain FK506-carboxymethoxyloximes at C22 has been performed as previously outlined by Kasper and co-workers [27] with some modifications (Scheme 1).



Scheme 1.

Briefly, to a solution of 0.061 mmol of FK506 in 10 mL of dry methanol, under argon, 0.34 mmol of anhydrous sodium acetate and 0.31 mmol of carboxymethoxylamine hemichlorhydrate (98%) were added. The mixture was stirred for 16 h while being monitored by silica gel TLC (CH_2Cl_2 –EtOAc–MeOH–HOAc, 30:70:11:0.6 per volume; iodine vapor as developer). Then the solvent was rotavaporated and a small amount of chloroform was added to partially dissolve the residue. The insoluble solid was a mixture of the reagent salts, while the organic phase contained exclusively the *Z/E* oxime products [27] as demonstrated by TLC and $^1\text{H-NMR}$ (200 MHz). The solid was then extracted several times with chloroform to yield 55 mg (100%) of FK506 oxime after rotavaporation of the combined extracts.

Activation of the FK506 carboxymethoxyloxime

The FK506 oxime (*Z/E* isomer mixture, 0.061 mmol) was dissolved in 5 mL of anhydrous dimethylformamide (DMF) (99.5%), and 0.12 mmol of DCC was added under argon. The mixture was stirred for 1.5 h. Complete disappearance of the oxime isomers was checked by silica gel TLC (CH_2Cl_2 –EtOAc–MeOH–HOAc, 30:70:11:0.6 per volume; iodine vapor as developer). The DCC-activated isomeric oximes produced a single spot in the TLC; their isolation was not attempted but they were used immediately for the next step.

Synthesis of the dansyl–FK506 (DNS-FK) conjugate

The DMF solution of the activated FK506 oxime from the previous step (0.061 mmol) was cannulated dropwise under argon over a solution of 0.108 mmol of dansylcadaverine in 2 mL of anhydrous dimethylformamide with stirring. After 72 h stirring at room temperature, the reaction mixture was heated at 90 °C under argon for 18 h while being monitored by silica gel TLC (*n*-PrOH–HOAc–water, 7:3:4 per volume). The solvent was then evaporated under vacuum and the resulting solid residue was dissolved in chloroform (50 mL) and extracted with 10% HCl solution (5×20 mL). After washing with water (3×20 mL), the organic extract was dried over anhydrous MgSO_4 and the solvent was then rotavaporated to yield, after vacuum drying (0.1 Torr), 52 mg of a yellow solid ($\lambda_{\text{max}}^{\text{abs}}$

333 nm in methanol). The solid was identified as the dansyl–FK506 conjugate by electrospray mass spectrometry (Bruker–Esquire LC), TLC analysis, and $^{13}\text{C-NMR}$ (Bruker; 50 MHz, CDCl_3).

Isolation of SP-A

Human SP-A was purified from surfactant isolated from alveolar proteinosis patients using sequential butanol and *n*-octylglucoside extractions as described elsewhere [7,28,29]. The purity of SP-A was checked by one-dimensional SDS–PAGE in 12% polyacrylamide gel under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 High Performance analyzer. The oligomerization state of SP-A was assessed by electrophoresis under nondenaturing conditions and electron microscopy as reported elsewhere [6,7,29]. SP-A isolated from alveolar proteinosis patients consisted of supratrimeric oligomers of at least 18 subunits.

Fluorescence measurements

Steady state fluorescence measurements were carried out using an Aminco AB2 spectrofluorimeter equipped with a thermostated cuvette holder (± 0.1 °C), using 5×5 -mm-pathlength quartz cuvettes. For DNS-FK emission intensity measurements, excitation was at 340 nm. Moreover, the background intensity in DNS-FK-free samples due to scattering by the proteins was subtracted from each recording of the fluorescence intensity.

Emission anisotropy

DNS-FK fluorescence emission anisotropy measurements were obtained with Glan prism polarizers. Excitation and emission wavelengths were set at 340 and 505 nm, respectively. For each sample, prepared as described above, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the excitation beam were collected 10 times each and then averaged. All measurements were performed at 25 °C. Anisotropy, *r*, was calculated as

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}, \quad (1)$$

where I_{\parallel} and I_{\perp} are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light, and G is the monochromator grating correction factor.

Binding studies

Fluorescence anisotropy titrations were performed in 5×5 -mm quartz cuvettes. Separate samples of $0.5 \mu\text{M}$ DNS-FK for each concentration of protein were prepared for each titration data point by diluting the stock DNS-FK solution to the desired final concentration in a total volume of 0.5 mL of 5 mM Tris-HCl, pH 7.4, buffer, containing 150 mM NaCl and 0.1 mM EDTA. An appropriate amount of SP-A, FK-BP, or HSA in the same buffer was added to each aliquot of DNS-FK to give the desired final concentration of protein, with less than a 5% increase in volume for each aliquot.

Data analysis

The interaction of DNS-FK with either SP-A, HSA, or FK-BP can be treated as a simple 1:1 association, with an apparent equilibrium constant given by

$$K_{app} = \frac{[FP]}{[F][P]}, \quad (2)$$

where $[FP]$ is the equilibrium concentration of the DNS-FK/protein complex, and $[P]$ and $[F]$ are the equilibrium concentrations of free protein and free DNS-FK, respectively. From the conservation of mass equations for protein and DNS-FK, $[F]$ and $[P]$ can be written as a function of the total molar concentrations of DNS-FK and protein, $[F]_T$ and $[P]_T$, respectively:

$$[F] = [F]_T - [FP] \quad \text{and} \quad (3)$$

$$[P] = [P]_T - [FP]. \quad (4)$$

Substitution of Eqs. (3) and (4) into Eq. (2) yields a quadratic equation for $[FP]$, whose relevant physical solution is given by

$$[FP] = \left\{ (1 + K_{app}([F]_T + [P]_T)) - \sqrt{(1 + K_{app}([F]_T + [P]_T))^2 - 4K_{app}^2[F]_T[P]_T} \right\} / 2K_{app}. \quad (5)$$

The steady state fluorescence anisotropy, r , measured at any point in an anisotropy titration, is represented as a sum of contributions from free and bound DNS-FK, each weighted according to their contribution to the total fluorescence intensity, as

$$r = f_{FP}r_{FP} + (1 - f_{FP})r_F, \quad (6)$$

where r_{FP} and r_F are the measured characteristic anisotropies of bound and free DNS-FK, respectively, and f_{FP} is the fraction of fluorescence intensity due to the bound form of DNS-FK,

$$f_{FP} = \frac{I_{FP}}{I_{FP} + I_F} = \frac{\varepsilon_{FP}\phi_{FP}[FP]}{\varepsilon_{FP}\phi_{FP}[FP] + \varepsilon_F\phi_F[F]}, \quad (7)$$

where ε_i and ϕ_i are the molar extinction coefficient and the fluorescence quantum yield of the i species. Substitution of Eqs. (3) and (4) into Eq. (7) yields

$$f_{FP} = \frac{[FP]S}{[F]_T + [FP](S - 1)}, \quad (8)$$

where S is a correction factor (equivalent to $\varepsilon_{FP}\phi_{FP}/\varepsilon_F\phi_F$) which accounts for experimental data being weighted in favor of the more fluorescent species.

Substituting Eqs. (8) and (5) into Eq. (6) yields an expression that relates the anisotropy values measured at any point of the titration to the apparent equilibrium binding constant:

$$r = \left(2K_{app}r_F[F]_T + (r_{FP}S - r_F) \left\{ [1 + K_{app}([F]_T + [P]_T)] - \sqrt{[1 + K_{app}([F]_T + [P]_T)]^2 - 4K_{app}^2[F]_T[P]_T} \right\} \right) / \left(2K_{app}[F]_T + (S - 1) \left\{ [1 + K_{app}([F]_T + [P]_T)] - \sqrt{[1 + K_{app}([F]_T + [P]_T)]^2 - 4K_{app}^2[F]_T[P]_T} \right\} \right). \quad (9)$$

Data from equilibrium binding titrations of DNS-FK were analyzed by nonlinear least-squares fitting to Eq. (9).

The Gibbs binding free energy was calculated from the K_{app} values according to the standard equation

$$\Delta G^0 = -RT \ln K_{app}, \quad (10)$$

where R is the general gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and T is the absolute temperature.

Fluorescence resonance energy transfer (FRET) assays

Fluorescence resonance energy transfer from SP-A tryptophan residues to the dansyl probe bound to FK506 was performed in 5 mM Tris-HCl, pH 7.4, buffer, containing 150 mM NaCl and 0.1 mM EDTA at 25 °C. Separate samples of $0.1 \mu\text{g/mL}$ of SP-A were titrated with appropriate amounts of DNS-FK to give the desired final concentration of drug, with less than a 5% increase in volume for each aliquot. Fluorescence emission intensity was recorded from 300 to 550 nm after excitation at 282 nm. The slit widths were 4 nm for the excitation and emission beams.

Results

Synthesis of DNS-FK

Drugs labeled with fluorescent reporter groups are used in studies on drug-protein interactions [13–16].

Dansyl was selected as fluorescent tag of FK506 given its low cost, its availability, the vast amount of data on its use as a biomolecule marker, and the significant sensitivity of its emission toward the microenvironment around the probe. Once a labeling protocol has been established for FK506, other fluorescent molecular probes with excitation in the visible spectrum may be easily introduced instead of dansyl, e.g., conveniently derivatized lucifer yellow or fluorescein. Although FK506 bears many different chemical groups (Fig. 1), its derivatization is neither a straightforward nor an easy task. Some of its functional groups are scarcely reactive, due to intrinsic factors (e.g., the carboxamide at C8) or steric hindrance (e.g., the OH group at C10), yet others would probably yield polyderivatization (such as OH at C24 or at the cyclohexane moiety or the two alkene groups). Still, chemical attachment of a “foreign” moiety to particular sites of FK506 would probably lead to significant or total loss of its activity. Kasper et al. [27] have recently described a procedure to conjugate FK506 to peptides via C22, a position that does not interfere with production of antibodies to it.

The FK506 derivatization procedure essentially involves its reaction with commercial carboxymethoxylamine to yield a mixture of the isomeric *Z/E* carboxymethoxyloximes at C22 [27]. In this way a carboxyl group is introduced in FK506 which eventually may be activated to allow reaction with nucleophiles (typically an amine group of derivatized dye molecules). The amide product is stable enough to let the conjugate survive under many experimental conditions and solvent media. A cadaverine spacer (five methylene groups plus the two amino end groups) should be long enough to allow a steric-hindrance-free binding of DNS-FK to proteins or any other interaction involving the drug molecule. The dansyl–FK506 conjugate was identified by electrospray mass spectrometry (Bruker–Esquire LC; methanol/acetonitrile/2% formic acid, positive ions detection; nominal mass for DNS-FK ($C_{63}H_{95}N_5O_{15}S$) = 1193; $[M + H]^+_{obs} = 1194$; $[M-DNS]^+ = 959$, base peak). The presence of a base peak in the ESI-MS spectrum was due to the loss of DNS via sulfonamide fragmentation [30]. By TLC analysis, DNS-FK came into view as a strong cyan fluorescent spot, $R_f = 0.89$, while the DNS fluorescence spot was weakly green with $R_f = 0.69$. Disappearance of the C22 carbonyl signal of FK506 at 212 ppm in ^{13}C -NMR spectra of DNS-FK (Bruker, 50 MHz, $CDCl_3$) was indicative of FK506 derivatization at C22.

Binding of DNS-FK to proteins

Fluorescence anisotropy is widely used for measuring high-affinity protein–protein or protein–nucleic acid interactions. The basis for the use of steady state anisotropy to assay ligand binding is that molecular rotations

that occur during the lifetime of the excited state of a fluorophore will depolarize the fluorescence, thereby providing an observable that is sensitive to molecular size. To assess the utility of DNS-FK as a fluorescent probe to measure the drug–protein interaction, we have determined the apparent equilibrium association constants of DNS-FK binding to FK-BP and HSA, to which FK506 binds [21–24]. In addition, we have used DNS-FK to evaluate the binding affinity of FK506 to SP-A.

Fluorescence intensity experiments were designed to determine whether DNS-FK binds to such proteins. The emission spectrum of 0.5 μ M DNS-FK in 5 mM Tris–HCl, 150 mM NaCl, 0.1 mM EDTA buffer, pH 7.4, was recorded after excitation at 340 nm, in the absence and presence of SP-A, FK-BP, and HSA (Fig. 2). In the absence of proteins, the fluorescence emission spectrum showed a maximum at 522 nm (Fig. 2A, continuous line). Addition of 0.5 μ M either FK-BP or SP-A resulted in a significant increase of fluorescence intensity, accompanied by a blueshift of the emission maximum from 522 to 492 nm for FK-BP (Fig. 2A, dashed–dotted line) and to 473 nm for SP-A (Fig. 2A, dashed line). This shift indicates a decrease of the polarity of the microenvironment around the fluorescent group upon binding to SP-A and FK-BP. Similar blueshifts have been reported for a variety of naphthalenesulfonate dyes in organic solvents or after being bound to proteins [18,31,32]. It is interesting to note that when 0.5 μ M HSA was added to DNS-FK, the fluorescence spectrum of the labeled drug redshifted to 537 nm, indicating a more polar environment of the drug with a slight enhancement of fluorescence intensity (Fig. 2A, dotted line).

To determine whether the changes observed in DNS-FK emission properties in the presence of SP-A, FK-BP, and HSA were due to the binding of these proteins to FK506 or to the fluorescent moiety of the labeled drug, we have studied the potential interaction between dan-

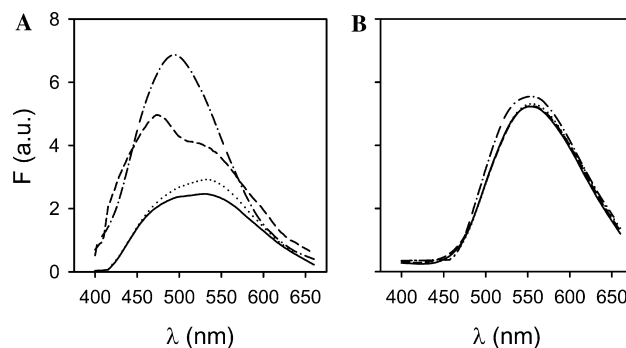


Fig. 2. Fluorescence emission spectra of (A) 0.5 μ M DNS-FK and (B) 0.5 μ M DNS in the absence (—) and the presence of 0.5 μ M SP-A (---), 0.5 μ M FK-BP (— · —), and 0.5 μ M HSA (·····) in 5 mM Tris–HCl, 150 mM NaCl, and 0.1 mM EDTA buffer, pH 7.4, at 25 °C. $\lambda_{ex} = 340$ nm. One representative experiment of three experiments is shown.

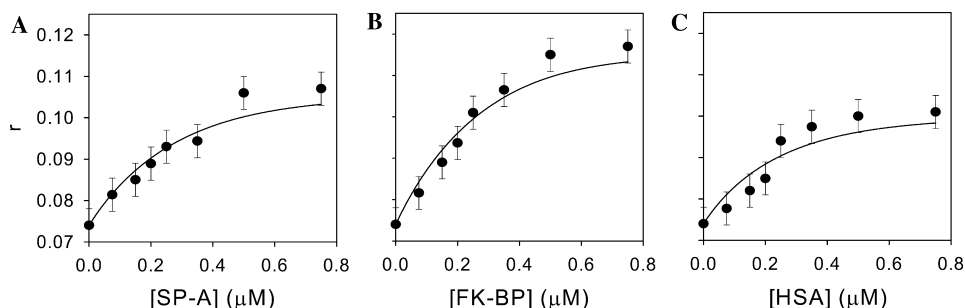


Fig. 3. Fluorescence anisotropy changes observed during titration of 0.5 μM DNS-FK with various proteins: (A) SP-A, (B) FK-BP, and (C) HSA. The data are means for three experiments. Solid lines are the results of nonlinear least-squares fitting using Eq. (9) as described under Materials and methods. $\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 505 \text{ nm}$; 25 $^{\circ}\text{C}$.

sylcadaverine and these proteins. Fig. 2B shows that addition of 0.5 μM SP-A, FK-BP, or HSA to 0.5 μM DNS did not affect, within experimental error, the fluorescence spectrum of the dye, which exhibited an emission maximum at 550 nm. Comparison of these results with those obtained with the labeled FK506 indicates that changes observed in the DNS-FK emission spectrum in the presence of SP-A, FK-BP, and HSA are actually due to the interaction of these proteins with the FK506 moiety.

Figs. 3A–C show the anisotropy data from titration of 0.5 μM DNS-FK with SP-A, FK-BP, and HSA. Before analyzing these data, it is important to recall that the bound DNS-FK has a higher fluorescence quantum yield than the free ligand, so that on a molar basis it makes a different contribution to the measured anisotropy. To correct for this bias, the characteristic anisotropy of the bound DNS-FK, r_{FP} , is weighted by the correction factor S . However, due to the fluctuations observed in the fluorescence intensity of DNS-FK in the presence of protein concentrations at which a saturation effect of the anisotropy values was observed, it was not possible to accurately measure S . These fluctuations might be due to the scatter contribution of the proteins, which becomes higher as the protein concentration is raised. This effect would be more important in the case of DNS-FK bound to HSA as the fluorescence intensity of the labeled drug bound to this protein is slightly higher than that of free DNS-FK. As the measured r -bound values were not affected by the uncertainty in the fluorescence intensity of bound DNS-FK, the r -bound value was fixed in the nonlinear least-squares analyses, and K_{app} and the S factor were the fitting parameters (Eq. 9). The correlation between K_{app} and S in all the fitting procedures was less than 0.001. The continuous lines in Fig. 3 represent the best nonlinear least-squares fit of the data to Eq. (9). The K_{app} and Gibbs free energy change (ΔG^0) values obtained for SP-A, FK-BP, and HSA from these fitting procedures are listed in Table 1. The standard Gibbs free energy change obtained for the interaction of DNS-FK with the three proteins indicates the spontaneity of the binding of DNS-FK with these pro-

Table 1

Apparent equilibrium association constants, K_{app} , and Gibbs free energy for DNS-FK binding to FK-BP, SP-A, and HSA

Protein	K_{app} (M^{-1})	ΔG^0 ($\text{kJ mol}^{-1} \text{K}^{-1}$)
FK-BP	$1.5 \times 10^7 \pm 0.1$	-41.0 ± 0.2
SP-A	$1.0 \times 10^7 \pm 0.1$	-40.0 ± 0.2
HSA	$1.0 \times 10^7 \pm 0.2$	-40.0 ± 0.5

teins. The K_{app} value for DNS-FK binding to SP-A, presented in Table 1, was calculated with regard to the molecular mass of the monomer (36 kDa). If the molecular mass of the octadecamer was considered (650 kDa), a K_{app} value of $(1 \pm 0.5) \times 10^8 \text{ M}^{-1}$ was obtained.

We also evaluated the impact of dansylation of FK506 at the C22 position on FK506 binding to FK-BP by determining the apparent decrease in binding resulting from addition of unmodified FK506. We found that addition of the nonemitting FK506 to a sample of DNS-FK bound to FK-BP led to a decrease of the observed anisotropy values of DNS-FK (data not shown). These results indicate that FK506 and DNS-FK compete for the drug-binding site in FK-BP and hence that the DNS-FK molecule binds to this protein by the FK506 moiety.

To ascertain whether the DNS-FK binding site in SP-A is located in the C-terminal domain of the protein, in which two conserved tryptophan residues, W191 and W213, are located, FRET assays were performed. The binding of SP-A to phospholipid vesicles was previously monitored as the fluorescence resonance energy signal from SP-A tryptophan residues (donor) to an appropriate acceptor such as a dansyl group located in the phospholipid surface [7]. Results indicate that no energy transfer from the tryptophan residues of SP-A to the dansyl moiety of DNS-FK was observed for the protein/drug molar ratios at which binding was determined (data not shown).

Discussion

We have prepared a fluorescent derivative of FK506 in which a dansyl moiety is covalently bound to the

C22 position of the FK506 molecule. This dansylated analogue of tacrolimus (DNS-FK) offers fluorescence properties for studying drug–protein assemblies. Its emission maximum and intensity change as a function of the solvent polarity, which allows its use to determine whether DNS-FK binds to proteins. In addition, changes in fluorescence anisotropy allow determination of the apparent equilibrium association constants of drug–protein complexes and the thermodynamics of such interactions. Thus we have studied the DNS-FK interaction with SP-A, using FK-BP and HSA as representative FK506-binding proteins.

To determine whether DNS-FK binds to SP-A, FK-BP, and HSA, we recorded its emission spectrum in the absence and presence of an equivalent concentration of each of these proteins. The spectral shifts and the increase in the amplitude of the fluorescence emission spectrum observed for DNS-FK in the presence of SP-A, FK-BP, and HSA indicated that the fluorescent analogue of FK506 binds to these three proteins. The lack of changes in the spectral properties of the precursor dye, dansylcadaverine, in the presence of either of the three proteins allowed us to infer that DNS-FK binds to SP-A, FK-BP, and HSA by the FK506 moiety. It is interesting to note that, whereas the DNS-FK fluorescence emission spectrum in the presence of FK-BP or SP-A shifted to lower wavelengths, a shift to higher wavelengths was observed upon addition of HSA. This redshift might be indicative of the interaction of the dansyl moiety of DNS-FK with polar amino acids of HSA such as Lys, Arg, or His located on the surface of the hydrophobic binding sites of the protein [33–35]. Thus, given the chemical structure of the hydrophobic DNS-FK molecule (Fig. 1), it is conceivable that the FK506 moiety was located in the hydrophobic cavity of HSA with the dansyl group on the surface of the hydrophobic pocket.

The formation of complexes between DNS-FK and either SP-A, FK-BP, or HSA led to an increase of fluorescence emission anisotropy, indicating that the binding of these proteins to DNS-FK caused mechanical restrictions of the rotational mobility of the dye. Addition of FK506 to DNS-FK/FK-BP complexes led to a reduction of the observed anisotropy values, approximating the anisotropy value of free DNS-FK. This indicates that FK506 and DNS-FK compete for the drug binding site in FK-BP and consequently that the DNS-FK molecule binds to this protein by the FK506 moiety. To determine the apparent equilibrium association constants for the interaction of DNS-FK with SP-A, FK-BP, and HSA, we measured the anisotropy of DNS-FK as a function of protein concentration. Fluorescence anisotropy titrations yielded a K_{app} value for the DNS-FK/SP-A complex of 10^7 M^{-1} , considering the molecular mass of the SP-A monomer (36 kDa), and $(1 \pm 0.5) \times 10^8 \text{ M}^{-1}$ if the molecular mass of the SP-A octadecamer was considered

(650 kDa). On the other hand, K_{app} for the DNS-FK/HSA complex was 10^7 M^{-1} . The apparent equilibrium association constant obtained for the DNS-FK/HSA complex is comparable to that obtained for the binding of other drugs, such as iodipamide [36] or norepinephrine [37], to HSA. K_{app} for the DNS-FK/FK-BP complex was $1.5 \times 10^7 \text{ M}^{-1}$, which is 164-fold weaker than that obtained by Bierer and co-workers [21] for the FK506/FK-BP assembly ($2.5 \times 10^9 \text{ M}^{-1}$) using a chromatography-based assay. These results indicate that derivatization of FK506 at the C22 position does not block, but partly inhibits, drug binding to FK-BP. Given that the region of FK506 which makes intimate contact with FK-BP is that extending from C24 to C15 (see Fig. 1), while the remainder of the molecule from C15 to C23 protrudes outward from the complex into the solvent [22,38], derivatization of FK506 at the C22 position should not affect drug binding to FK-BP. We suggest that the partial inhibitory effect of the dansyl tag on FK506 binding affinity can be explained by a possible collapse of the dansyl moiety under the FK506 macrocyclic ring due to the hydrophobicity of the dye in aqueous solution. This possible interaction between the FK506 and the appending dye is inferred from the blueshift in the emission maximum of DNS-FK (522 nm) compared with that of dansylcadaverine (550 nm), which indicates a decrease in the polarity of the environment of the probe.

Given that FK506 has been modified at the C22 position, it is likely that the C24 to C15 region of FK506 is also involved in drug binding to SP-A and HSA. Binding of FK506 to extracellular fluid proteins such as HSA (mainly present in serum) or SP-A (present in the alveolar fluid) might result in increased FK506 solubility in plasma or in alveolar fluid, decreased toxicity, and/or protection against oxidation of the bound ligand. Binding can also have a significant impact on the pharmacokinetics of the drug.

To determine whether the DNS-FK binding site in SP-A was located in the C-terminal domain of the protein, in which the major lipid-binding site is positioned near two conserved tryptophan residues, W191 and W213 [7], we performed fluorescence resonance energy transfer assays. No energy transfer from the tryptophan residues of SP-A to the dansyl moiety of DNS-FK was observed for the protein/drug molar ratios at which binding was determined. Assuming a Förster distance, R_0 , for the tryptophan–dansyl (donor–acceptor) pair of 21 Å [39] and considering the dependence of energy transfer efficiency on distance, we found that the DNS-FK binding site should be located at a distance of at least $2R_0$ from the tryptophan residues, which would correspond to the neck region of SP-A in the crystal structure of the protein determined by Head and co-workers [40] or beyond. Thus the tacrolimus-binding site might be located in the neck region, in the collagen-like domain, or in the N-terminal segment of

SP-A. Considering that the latter consists of only 7–10 amino acids and that intertriple helix interactions of the collagen-like domain appear to be mediated primarily by salt bridging, van der Waals forces, and electrostatic interactions, it is conceivable that the drug-binding site could be located in the neck region of the protein, which consists of an amphipathic α helix that forms a coiled-coil with the amphipathic α helices of two other monomers [40]. However, we cannot exclude the hypothesis that the absence of demonstrable energy transfer from the tryptophan residues of SP-A to the dansyl moiety of DNS-FK may be due to the possibility that the excitation transition dipole of the dansyl group may be perpendicular, or nearly perpendicular, to the emission dipoles of the tryptophan residues.

Future studies will determine the consequences of the interaction of FK506 with SP-A on the immunosuppressive properties of FK506 and on the diverse functions of SP-A. SP-A itself has antiinflammatory and immunosuppressive activities in the lung [7–9]. Thus further studies analyzing whether FK506 and SP-A together interfere with the antiinflammatory and immunosuppressive action of each alone or, on the contrary, jointly have a greater inhibitory effect than either alone on the release of proinflammatory cytokines by lung macrophages and lymphocytes should be performed.

References

- [1] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishimaya, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physico-chemical and biological characteristics, *J. Antibiot.* 40 (1987) 1249–1255.
- [2] J. Clardy, The chemistry of signal transduction, *Proc. Natl. Acad. Sci. USA* 92 (1995) 56–61.
- [3] L.J. Scott, K. McKeage, S.J. Keam, G.L. Plosker, Tacrolimus, a further update of its use in the management of organ transplantation, *Drugs* 63 (2003) 1247–1297.
- [4] L.M.G. Van Golde, C. Casals, Metabolism of lipids in the lung: scientific foundations, in: R.G. Crystal, J.B. West (Eds.), second edition, Lippincott-Raven Publishers, Philadelphia, 1997, pp. 9–18.
- [5] J. Goerke, Pulmonary surfactant: functions and molecular composition, *Biochim. Biophys. Acta* 1408 (1998) 79–89.
- [6] T. Voss, H. Eistetter, K.P. Schafer, J. Engel, Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36. Structural homology with the complement factor C1q, *J. Mol. Biol.* 201 (1988) 219–227.
- [7] F. Sanchez-Barbero, J. Strassner, R. Garcia-Canero, W. Steinhilber, C. Casals, Role of the degree of oligomerization in the structure and function of human surfactant protein A, *J. Biol. Chem.* 280 (2005).
- [8] F.X. McCormack, J.A. Whitsett, The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung, *J. Clin. Invest.* 109 (2002) 707–712.
- [9] H. Sano, Y. Kuroki, The lung collectins, SP-A and SP-D, modulate innate immunity, *Mol. Immunol.* 42 (2005) 279–287.
- [10] A.M. LeVine, J.A. Whitsett, Pulmonary collectins and innate host defense of the lung, *Microbes Infect.* 3 (2001) 161–166. Review.
- [11] C. Casals, Role of surfactant protein A (SP-A)/lipid interactions for SP-A functions in the lung, *Pediatr. Pathol. Mol. Med.* 20 (2001) 249–268.
- [12] O. Cañadas, R. Guerrero, R. García-Cañero, G. Orellana, M. Menéndez, C. Casals, Characterization of liposomal tacrolimus in lung surfactant-like phospholipids and evaluation of its immunosuppressive activity, *Biochemistry* 43 (2004) 9926–9938.
- [13] M.P. Lillo, O. Cañadas, R.E. Dale, A.U. Acuña, The location and properties of the taxol binding center in microtubules: a picosecond laser study with fluorescent taxoids, *Biochemistry* 41 (2002) 12436–12449.
- [14] Y. Li, R. Edsall, P.G. Jagtap, D.G. Kingston, S. Bane, Equilibrium studies of a fluorescent paclitaxel derivative binding to microtubules, *Biochemistry* 39 (2000) 616–623.
- [15] M. Galleni, B. Lakaye, S. Lepage, M. Jamin, I. Thamm, B. Joris, J.M. Frere, A new, highly sensitive method for the detection and quantification of penicillin-binding proteins, *Biochem. J.* 291 (1993) 19–21.
- [16] B. Lakaye, C. Damblon, M. Jamin, M. Galleni, S. Lepage, B. Joris, J. Marchand-Brynaert, C. Frydrych, J.M. Frere, Synthesis, purification and kinetic properties of fluorescein-labelled penicillins, *Biochem. J.* 300 (1994) 141–145.
- [17] L. Stryer, The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites, *J. Mol. Biol.* 13 (1965) 482–495.
- [18] W.O. McClure, G.M. Edelman, Fluorescent probes for conformational states of proteins. I. Mechanism of fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate, a hydrophobic probe, *Biochemistry* 5 (1966) 1908–1919.
- [19] W.O. McClure, G.M. Edelman, Fluorescent probes for conformational states of proteins. II. The binding of 2-*p*-toluidinylnaphthalene-6-sulfonate to alpha-chymotrypsin, *Biochemistry* 6 (1967) 559–566.
- [20] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, second ed., Kluwer, New York, 1999.
- [21] B.E. Bierer, P.S. Mattila, R.F. Standaert, L.A. Herzenberg, S.J. Burakoff, G. Crabtree, S.L. Schreiber, Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin, *Proc. Natl. Acad. Sci. USA* 87 (1990) 9231–9235.
- [22] G.D. van Duyn, R.F. Standaert, P.A. Karplus, S.L. Schreiber, J. Clardy, Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex, *Science* 252 (1991) 839–842.
- [23] G. Wiederrecht, E. Lam, S. Hung, M. Martin, N. Sigal, The mechanism of action of FK-506 and cyclosporin A, *Ann. N. Y. Acad. Sci.* 696 (1993) 9–19.
- [24] R. Bakhtiar, R.A. Stearns, Studies on non-covalent associations of immunosuppressive drugs with serum albumin using pneumatically assisted electrospray ionization mass spectrometry, *Rapid Commun. Mass Spectrom.* 9 (1995) 240–244.
- [25] A.J. Beysens, G.H. Beuman, J.J. Vander Heijden, K.E.J. Hoogtanders, O.M. Steijger, H. Lingeman, Determination of tacrolimus (FK-506) in whole-blood using liquid-chromatography and fluorescence detection, *Chromatographia* 39 (1994) 490–496.
- [26] K. Hane, M. Fujioka, Y. Namiki, T. Kitagawa, N. Kihara, K. Shimatani, T. Yasuda, Physico-chemical properties of (–)-(1*R*, 9*S*, 12*S*, 13*R*, 14*S*, 17*R*, 18*E*, 21*S*, 23*S*, 24*R*, 25*S*, 27*R*)-17-allyl-1, 14-dihydroxy-12-[(*E*)-2-[(1*R*, 3*R*, 4*R*)-4-hydroxy-3-methoxy cyclohexyl]-1-methylvinyl]-23, 25-dimethoxy-13, 19, 21, 27-tetramethyl-11, 28-dioxo-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2, 3, 10, 16-tetrone hydrate (FK-506), *Iyakuhi Kenkyu* 23 (1992) 33–43.
- [27] K.C. Kasper, H. Jeong, D. Davalian, H.-T. Liu, P.L. Miller, D.L. Williams, Monoclonal antibodies to tacrolimus and immunoassay methods for tacrolimus, PCT Patent WO01/09190 (2001).
- [28] C. Casals, E. Miguel, J. Perez-Gil, Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles, *Biochem. J.* 296 (1993) 585–593.

- [29] I. Garcia-Verdugo, F. Sanchez-Barbero, F.U. Bosch, W. Steinhilber, C. Casals, Effect of hydroxylation and N187-linked glycosylation on molecular and functional properties of recombinant human surfactant protein A, *Biochemistry* 42 (2003) 9532–9542.
- [30] C. Cavaliere, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, A simple and sensitive liquid-chromatography-mass spectrometry confirmatory method for analyzing sulfonamide antibacterials in milk and egg, *J. Agric. Food Chem.* 51 (2003) 558–566.
- [31] R.R. Hantgan, Steady-state polarization of dansylcadaverine-fibrinogen: evidence for flexibility, *Biochemistry* 21 (1982) 1821–1829.
- [32] I. Plasencia, A. Cruz, C. Casals, J. Perez-Gil, Superficial disposition of the N-terminal region of the surfactant protein SP-C and the absence of specific SP-B-SP-C interactions in phospholipid bilayers, *Biochem. J.* 359 (2001) 651–659.
- [33] Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, *Protein Eng.* 12 (1999) 439–446.
- [34] X.M. He, D.C. Carter, Atomic structure and chemistry of human serum albumin, *Nature* 358 (1992) 209–215.
- [35] K.J. Fehske, W.E. Muller, U. Wollert, The location of drug binding sites in human serum albumin, *Biochem. Pharmacol.* 30 (1981) 687–692.
- [36] K. Yamasaki, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Characterization of site I on human serum albumin: concept about the structure of a drug binding site, *Biochim. Biophys. Acta* 1295 (1996) 147–157.
- [37] W.J. Jusko, U. Gretch, Plasma and tissue protein binding of drugs in pharmacokinetics, *Drug Metab. Rev.* 5 (1976) 43–140.
- [38] M.T. Ivery, L. Weiler, Modeling the interaction between FK506 and FKBP12: a mechanism for formation of the calcineurin inhibitory complex, *Bioorg. Med. Chem.* 5 (1997) 217–232.
- [39] P. Wu, L. Brand, Resonance energy transfer: methods and applications, *Anal. Biochem.* 218 (1994) 1–13.
- [40] J.F. Head, T.R. Mealy, F.X. McCormack, B.A. Seaton, Crystal structure of trimeric carbohydrate recognition and neck domains of surfactant protein A, *J. Biol. Chem.* 278 (2003) 43254–43260.