Antibody–Stilbene Complexes

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The Effects of Antibodies on Stilbene Excited-State Energetics**

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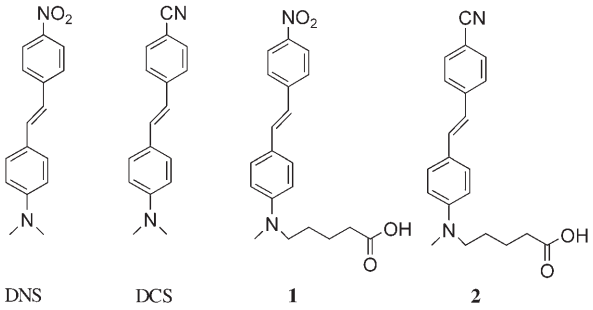
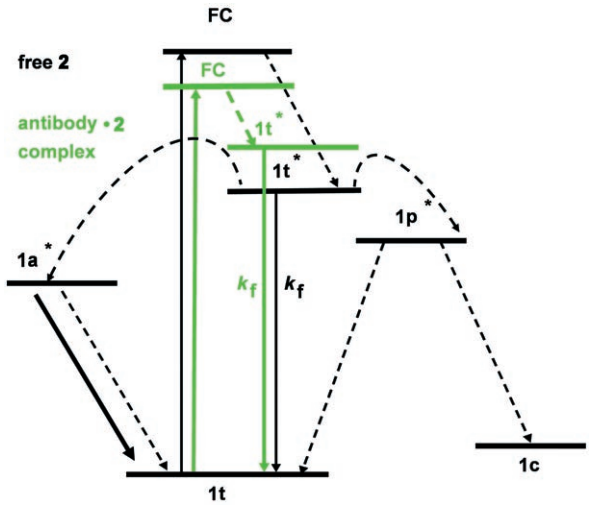


DNS DCS **1** **2**

Antibodies can be used to control the energetics and reactions of molecules in their ground states as well as in their electronically excited states.[1] For example, antibodies have been generated that catalyze the light-dependent cleavage of thymine dimers, and that modulate the excited-state behavior of *trans*-stilbene.[2,3] In particular, Lerner and co-workers generated a blue-fluorescent antibody, 19G2, which binds *trans*-stilbene to give a strongly fluorescent complex. Detailed structural and photophysical studies revealed that the antibody stabilizes the planar excited-state configuration through the formation of an exciplex between Trp^{H103} of the antibody and stilbene. To further explore the effects of the well-defined environments afforded by antibody binding sites on excited-state energy surfaces, we have generated antibodies to the donor–acceptor-substituted stilbene *trans*-4-*N*,*N*-dimethylamino-4′-cyanostilbene (DCS).

Unlike the widely accepted two-state model of photophysical unsubstituted *trans*-stilbene (Scheme 1), which consists of an initial emissive $^1t^*$ state and a subsequently populated nonemissive $^1p^*$ state (which is twisted by 90° about the C=C bond and undergoes internal conversion to give a mixture of *trans*- and *cis*-stilbene[4]), the photophysics of donor–acceptor-substituted stilbenes are more complicated.[5] A number of experimental and theoretical results have been interpreted in terms of an additional excited state, $^1a^*$, which corresponds to a relatively low-lying, twisted intramolecular charge-transfer (TICT) state in which the styryl-anilino C–C bond is rotated (Scheme 1).[6–8] Others have questioned the necessity of a TICT state and have suggested that the photophysics of substituted DCS analogues can be explained with a two-state model.[9–11]

Monoclonal antibodies (Mabs) provide a unique opportunity to study the photophysics of DCS in distinct steric and electronic enviroments without introducing any substituents. To this end, antibodies were generated against the DCS derivative **2**, as well as *trans*-4-*N*,*N*-dimethylamino-4′-nitrostilbene (DNS) derivative **1**, an analogue with a larger dipole moment (7.42 D)[13] which might more closely approximate



Scheme 1. Excited-state photochemistry of free **2** (black) and hypothesized primary pathway of the antibody–**2** complexes (green). Dashed lines: nonradiative transition; solid lines: radiative transitions. FC = Franck–Condon excited state.

the polar excited state of DCS. These antibody–stilbene complexes are highly fluorescent and emit blue to green light. Mabs 16E3, 17E3, and 17A4 elicited against hapten **2** and Mab 11G10 raised against the more polar hapten **1** were spectroscopically characterized (Table 1). The absorption

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Table 1: Steady-state spectral data and affinities for haptens **1** and **2**, antibody–**2** complexes, and the 11G10–**1** complex.

Hapten and complex	λ_{abs} [nm]	λ_{em} [nm]	$\varepsilon_{max}\times10^{-3}$ [$M^{-1}cm^{-1}$]	Stokes shift [cm^{-1}]	Φ_f	K_d [nM]
1	430	[a]	7.30	[a]	0	[a]
11G10–**1**	532	684	9.45	4177	0.002	13
2	373	574	14.1	9388	0.16	[a]
11G10–**2**	424	515	23.0	4168	0.32	23
16E3–**2**	410	490	16.4	3982	0.60	30
17A4–**2**	412	505	14.4	4470	0.64	50
17E3–**2**	396	475	14.6	4200	0.57	75

[a] Not applicable.

spectra of free **2** and antibody–**2** complexes display no substructure (Figure 1). While free **2** absorbs with a λ_{max} of 373 nm, the absorption maxima of antibodies 16E3, 17E3, and

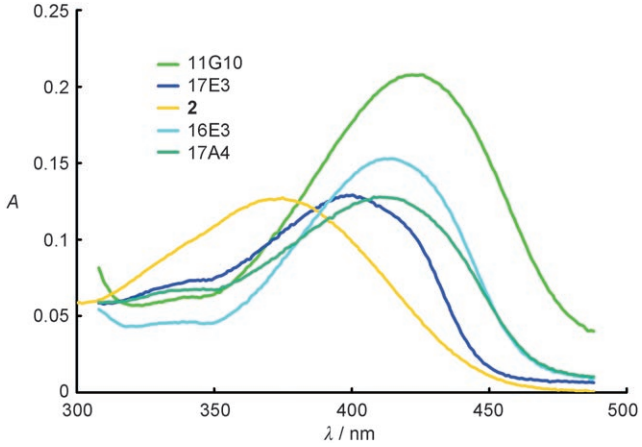

Figure 1. Steady-state absorption spectra of **2** (10 μM) and antibody–**2** complexes (10 μM) in phosphate-buffered saline (PBS) containing 5% DMF at room temperature.

17A4 complexed with **2** are red-shifted by 23–40 nm. When **2** is bound to antibody 11G10, the absorption maximum is red-shifted by 51 nm. This shift is consistent with the notion that 11G10, which was generated to the more polar DNS analogue **1**, is more complementary to the highly polar Franck–Condon (FC) excited state of DCS. The significant red-shifts of all antibody–stilbene complexes suggest that preorganized antibody combining sites are more effective in stabilizing the FC excited states of **1** and **2** than aqueous bulk solvent, which cannot reorganize to solvate the more polar excited state on the timescale of light absorption.

The steady-state fluorescence spectra of **2** and antibody–**2** complexes are also broad and devoid of any substructure. In contrast to the situation in bulk solution, in which the band widths at half-maximum increase with solvent polarity,[9] band widths at half-maximum for the antibody–**2** complexes are narrower than those of free **2**, suggesting that some rotatory conformations of **2** are constrained in the antibody combining sites. As expected for a stilbene molecule with an increased activation energy of rotation around the C=C bond in the excited state, the fluorescence quantum yields (Φ_f) of the antibody–**2** complexes are two to four times higher than that of free **2**; the rate constants for nonradiative decay, k_{nr}, obtained from time-resolved measurements decrease correspondingly (Table 2). All of the antibody–**2** complexes have large blue-shifts (59–99 nm) with respect to free **2** (Figure 2) and emit light of distinct colors ranging from blue for the 17E3–**2** complex (475 nm) to green for the 11G10–**2** complex (515 nm).

Table 2: Results of time-resolved fluorescence spectroscopy of **2** and antibody–**2** complexes.

Hapten and complex	τ_f [ns]	$\tau_r=\tau_f/\Phi_f$ [ns]	$k_r=1/\tau_r$ [ns^{-1}]	$k_{nr}=1/\tau_f-k_r$ [ns^{-1}]
2	1.01	6.3	0.16	0.83
11G10–**2**	2.68	8.4	0.12	0.25
16E3–**2**	2.78	4.6	0.22	0.14
17A4–**2**	2.88	4.5	0.22	0.13
17E3–**2**	2.53	4.4	0.23	0.17

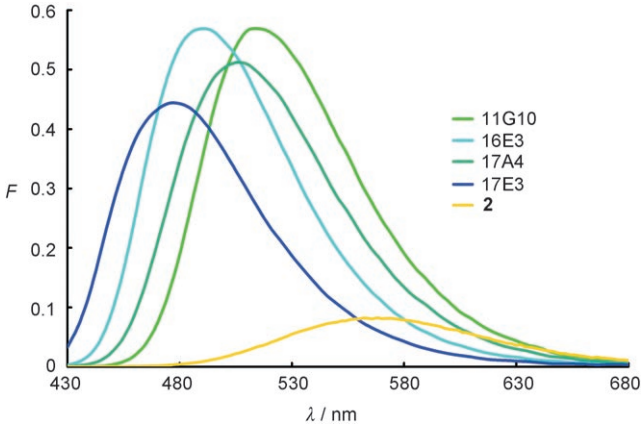

Figure 2. Steady-state fluorescence spectra of **2** (10 nM) and antibody–**2** complexes (10 nM) in PBS containing 5% DMF at room temperature. The λ_{abs} values listed in Table 1 were used as λ_{ex} for each corresponding spectrum.

The overall blue-shift in fluorescence of the antibody–**2** complexes relative to **2** in water can be ascribed to the preorganized nature of antibody combining sites. Bulk solvent has sufficient time to reorganize to preferentially stabilize the polar excited $^1t^*$ state of **2** relative to the less polar ground state prior to radiative decay. On the other hand, although the antibody binding site is more complementary to the polar excited state of DCS than to the ground state, it cannot reorganize to the same degree as bulk water, resulting in the large blue-shifts in fluorescence of the antibody–**2** complexes. Alternatively, if we assume that free **2** relaxes to an emissive low-lying TICT state ($^1a^*$), destabilization of the transition state by the antibody would lead to a switch to $^1t^*$ as the emissive state of the antibody–**2** complexes, which would equally well explain the blue-shift in fluorescence. The relative ordering of the antibodies with respect to the increasing emission maxima is the same ordering as for increasing absorption maxima (Table 1). This finding is consistent with a similar underlying mechanism for the two

events, that is, stabilization of the FC excited state (absorption) and the emitting state (fluorescence) by the antibodies through electrostatic and van der Waals interactions.

To characterize the nature of an antibody–stilbene complex and its effect on the photophysics of DCS in atomic detail, we have determined the crystal structure of Fab 11G10 in complex with hapten **1** at 2.75-Å resolution (Figure 3, for details see the Supporting Information). The stilbene moiety is bound in a planar conformation. Rotation around the excited-state C=C bond as well as the styryl-anilino C–C bond are likely to be restricted by interactions between the ligand and antibody residues Phe^{L94}, Leu^{L89}, Leu^{L36}, Tyr^{H33}, Tyr^{H95}, and Val^{H93} (Figure 3c). The binding pocket possesses a relatively high percentage of polar residues in immediate vicinity to the ligand. The bottom of the cavity exhibits a positive electrostatic potential (Figure 3b) due to the positively charged guanidinium group of Arg^{L46} (Figure 3a,c). Towards the mouth of the cavity, the electrostatic potential becomes partially negative. Importantly, the charge distribution in the combining site together with the aromatic ring systems of Tyr^{H33}, Tyr^{H95}, and Phe^{L94} are well suited to stabilize charge separation in the excited state of DCS derivative **2**.

In summary, we have generated antibodies that bind donor–acceptor-substituted stilbenes to afford strong blue to green fluorescent complexes. Spectroscopic and structural data suggest a model in which the relatively rigid protein environment sterically increases the activation barriers connecting the planar $^1t^*$ to the twisted excited states $^1p^*$ (and possibly $^1a^*$) and modulates the energetics of the fluorescent $^1t^*$ state (Scheme 1). The antibodies appear to be more effective in stabilizing the polar FC excited state of **2** relative to the ground state, but less effective than bulk water in selectively stabilizing the light-emitting state. In practical terms, the relatively long excitation and emission wavelengths of the antibody–**2** complexes may make them useful for in vitro and in vivo applications as fluorescent biosensors.

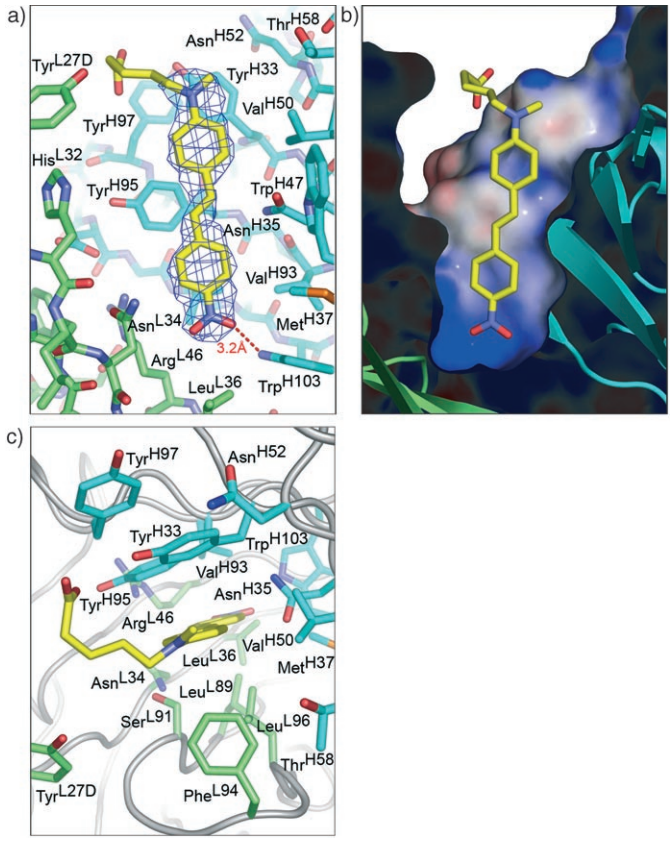

Figure 3. Antibody combining site of 11G10 in complex with hapten **1** with electron density contoured at 1.5 σ. a) A slice through the center of the antibody combining site is shown. The only hydrogen bond between **1** and 11G10 is illustrated as a red dashed line. b) Representation of the electrostatic surface; similar view to that in (a). c) View of the antibody binding pocket rotated horizontally by 90° to the view in (a).

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