

Understanding the Variable Quenching of Tryptophan Fluorescence in Proteins: Modulation of Electron Transfer rates by electrostatics

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Acknowledgements

James T. Vivian, Postdoctoral Associate 2000-2002

Tiqing Liu, Postdoctoral Associate 2003-2005

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This Power Point presentation is based on the following publications:

- T. Liu et al, [Ionization Potentials of Fluoroindoles and the Origin of Non-Exponential Tryptophan Fluorescence Decay in Proteins](#), *J. Am. Chem. Soc.* **127**, 4104-4113 (2005)
- P. R. Callis and T. Liu, [Quantitative Prediction of Fluorescence Quantum Yields for Tryptophan in Proteins](#), *J. Phys. Chem. B* **108**, 4248-4259 (2004)
- P. R. Callis and J. T. Vivian, *Chem. Phys. Letters* **369**, 409-414 (2003).
- J.T. Vivian and P.R. Callis, *Biophysical J.* **80**, 2093-2109 (2001)

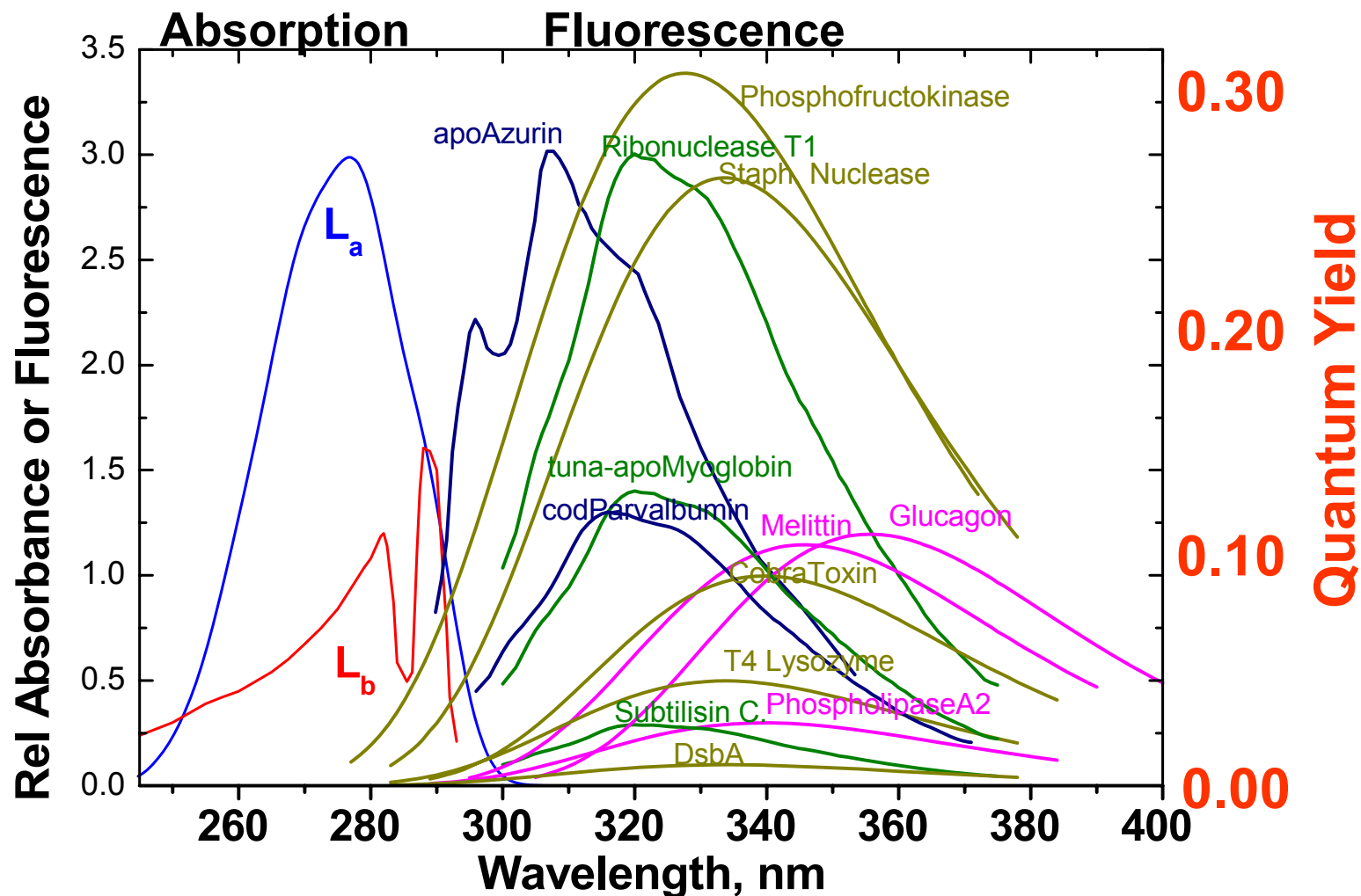
Tryptophan (Trp) fluorescence intensity (quantum yield) and fluorescence lifetime is highly sensitive to local protein environment. Figure 1 shows the Trp fluorescence spectra from a selection of single-Trp proteins, with the amplitude proportional to reported quantum yields.

~200 papers/year are published containing results that use Trp fluorescence intensity changes to study protein folding, ligand binding, protein-protein interactions, etc.

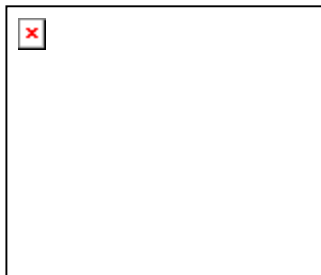
Trp is still the most reliable fluorescent probe of protein structure and dynamics because it is intrinsic. There are no uncertainties regarding perturbation of structure that are typically associated with the use of artificial non-intrinsic probes.

Our work is based on the premise that Trp would be MUCH more useful if we understood the mechanism of the intensity variations.

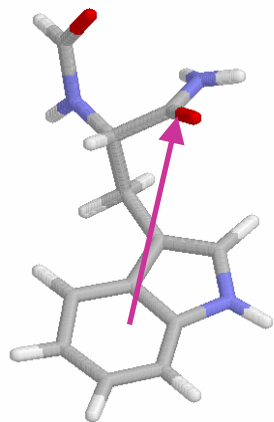
Figure 1. The 30-fold variations in fluorescence quantum yield and lifetime of tryptophan in proteins have been a great puzzle for nearly 40 years.



Puzzling because 3MI



Amide group implicated
for over 30 years



has quantum yield ~ 0.30
 \sim *independent* of solvent polarity

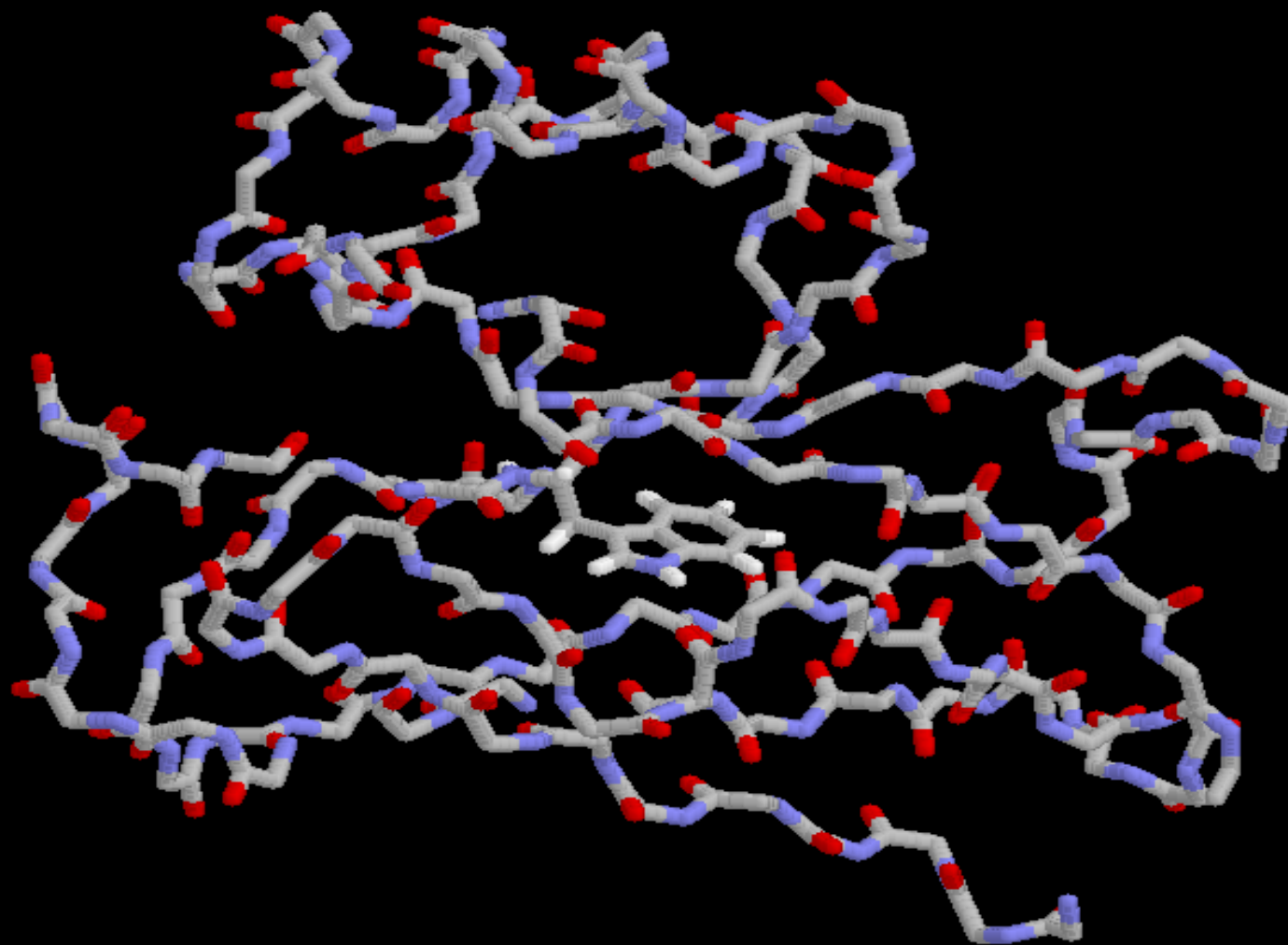
NATA

Has quantum Yield = 0.14 in water;
quantum yield = 0.4 in dioxane
(P. Muino & P. Callis, in prep.)

Recent work by the Barkley, Engelborghs, and Bombarda groups has strongly reinforced the long-held belief that **electron transfer to the local backbone amides causes quenching in proteins.**

But, every Trp in a protein is surrounded by amides. Why do several Trps in proteins exhibit quantum yields = 0.3 , while others exhibit much smaller yields (down to 0.01 or less)???

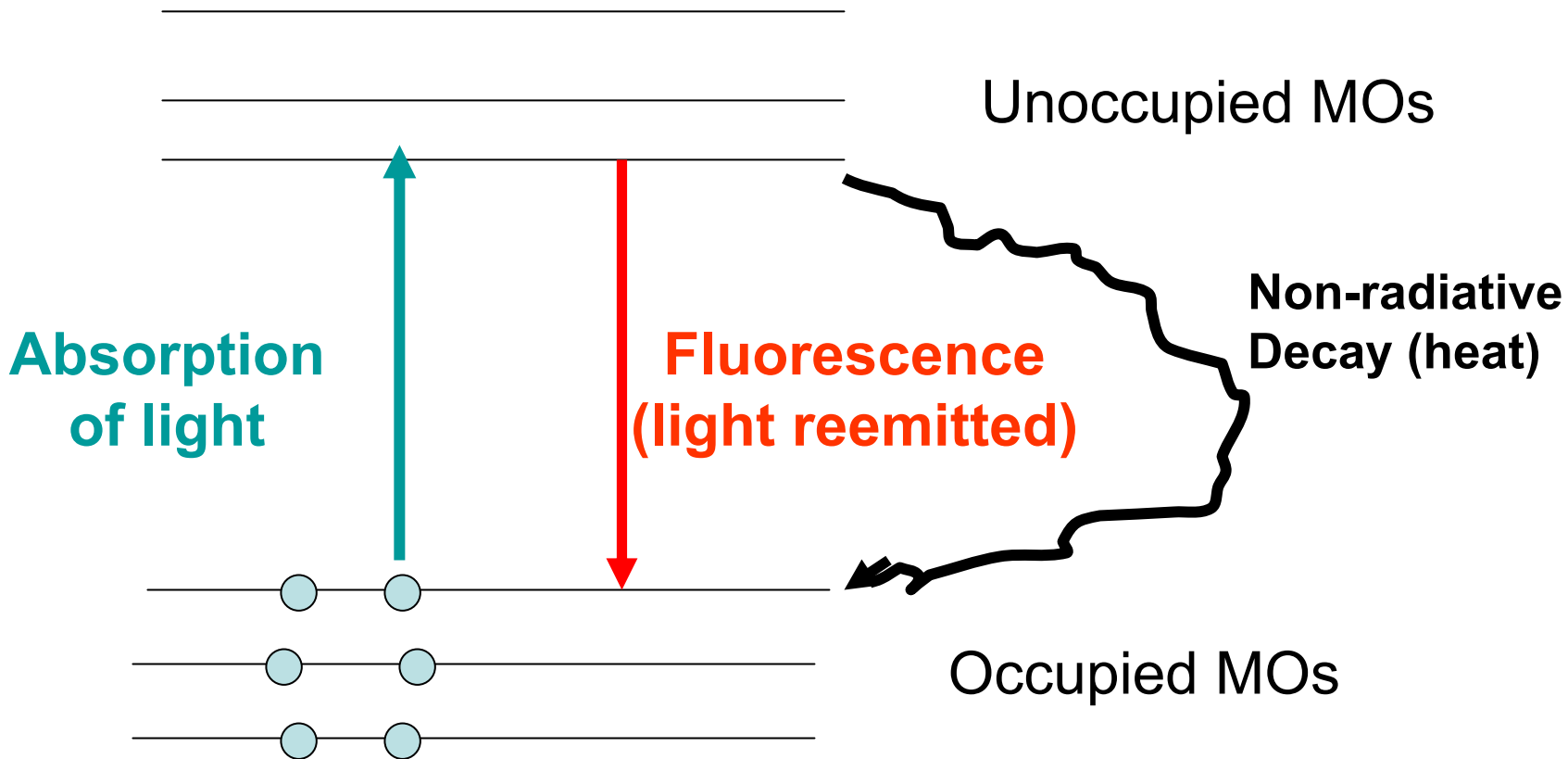
Trp48 of azurin has quantum yield = 0.3



The amide group is not a strong electron acceptor.

We investigated the hypothesis that local electric fields might stabilize charge transfer to a nearby amide in some proteins but not in others.

In this view, variability of the Trp fluorescence yield is dictated by variations in the electron transfer rate, k_{ET} .



Fluorescence Quantum Yield = Photons emitted / Photons absorbed

= Radiative rate / (Radiative rate + Non-radiative rate)

= $k_{\text{rad}} / (k_{\text{rad}} + k_{\text{nr}} + k_{\text{ET}})$ ← variability comes from **electron transfer rate constant**

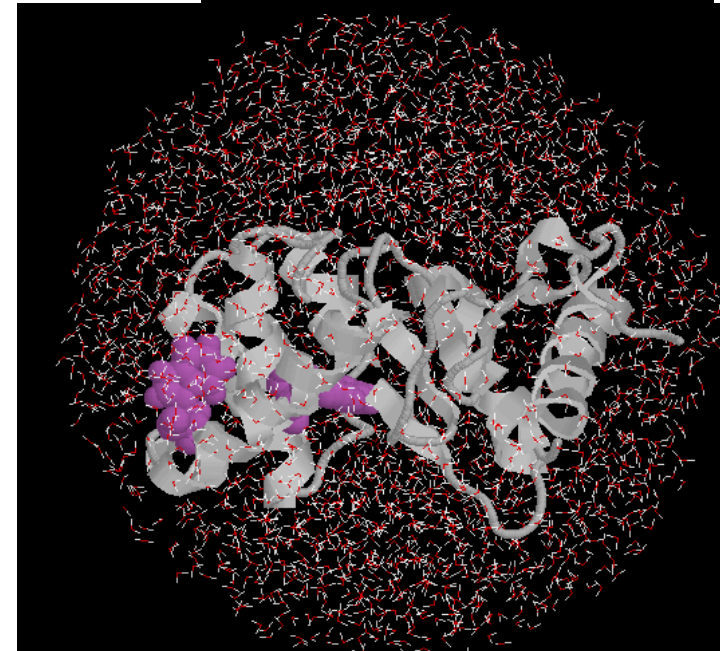
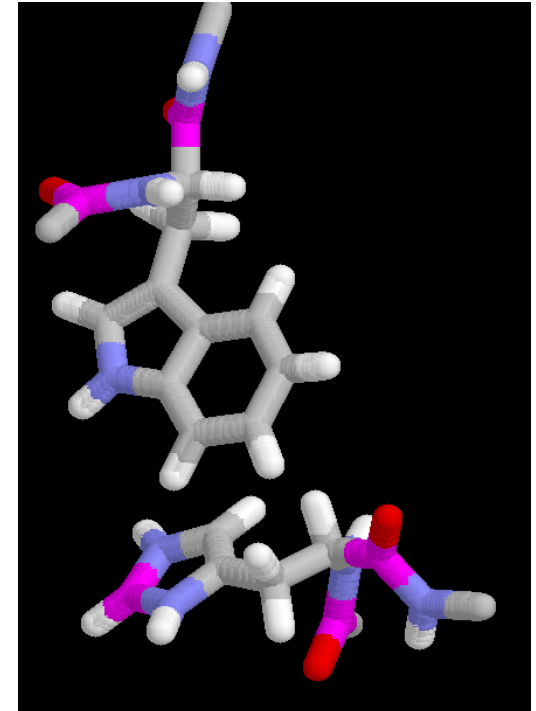
METHODS:

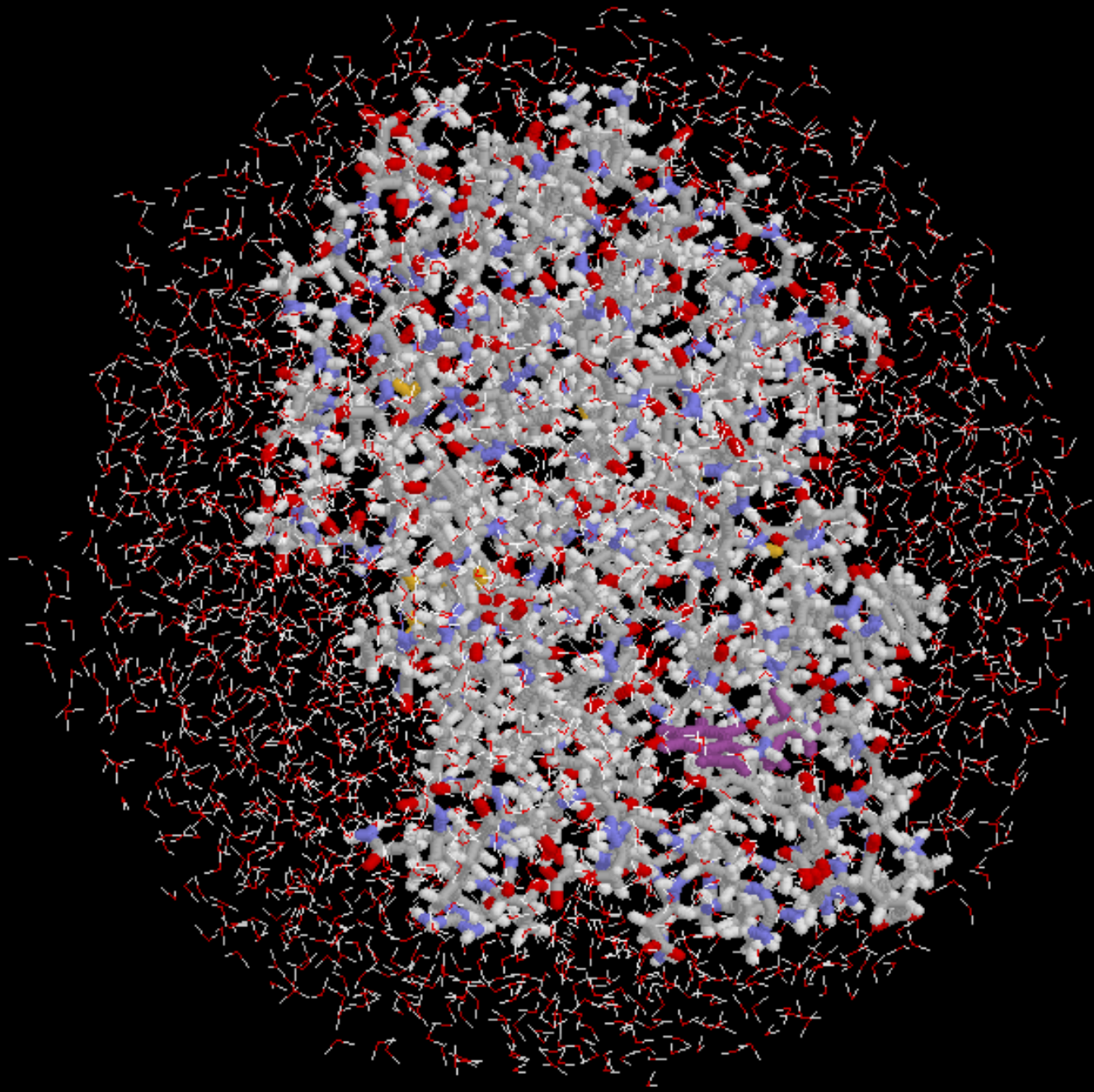
Zerner's INDO/S-CIS semiempirical QM for the "chromophore", which is generally a two-residue supermolecule of the N-formylamides.

Includes electric potential and fields at each atom of the chromophore from the Coulomb sum over all point charges representing the protein and water from the MD (CHARMM).

QM charges are fed back to the MD every 10 fs.

Xray or NMR structure ----->
+ water sphere





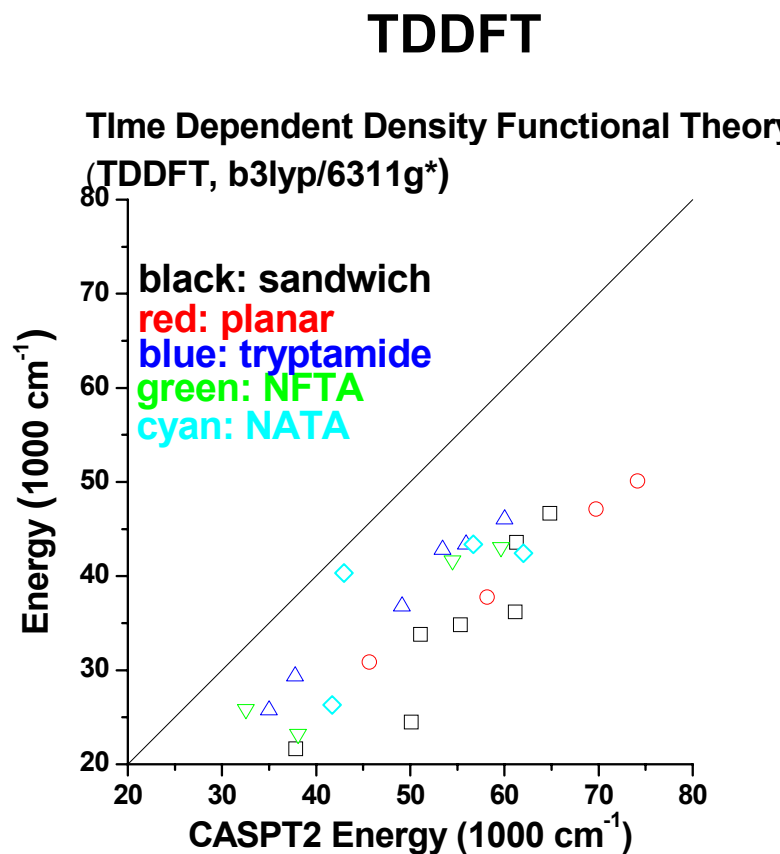
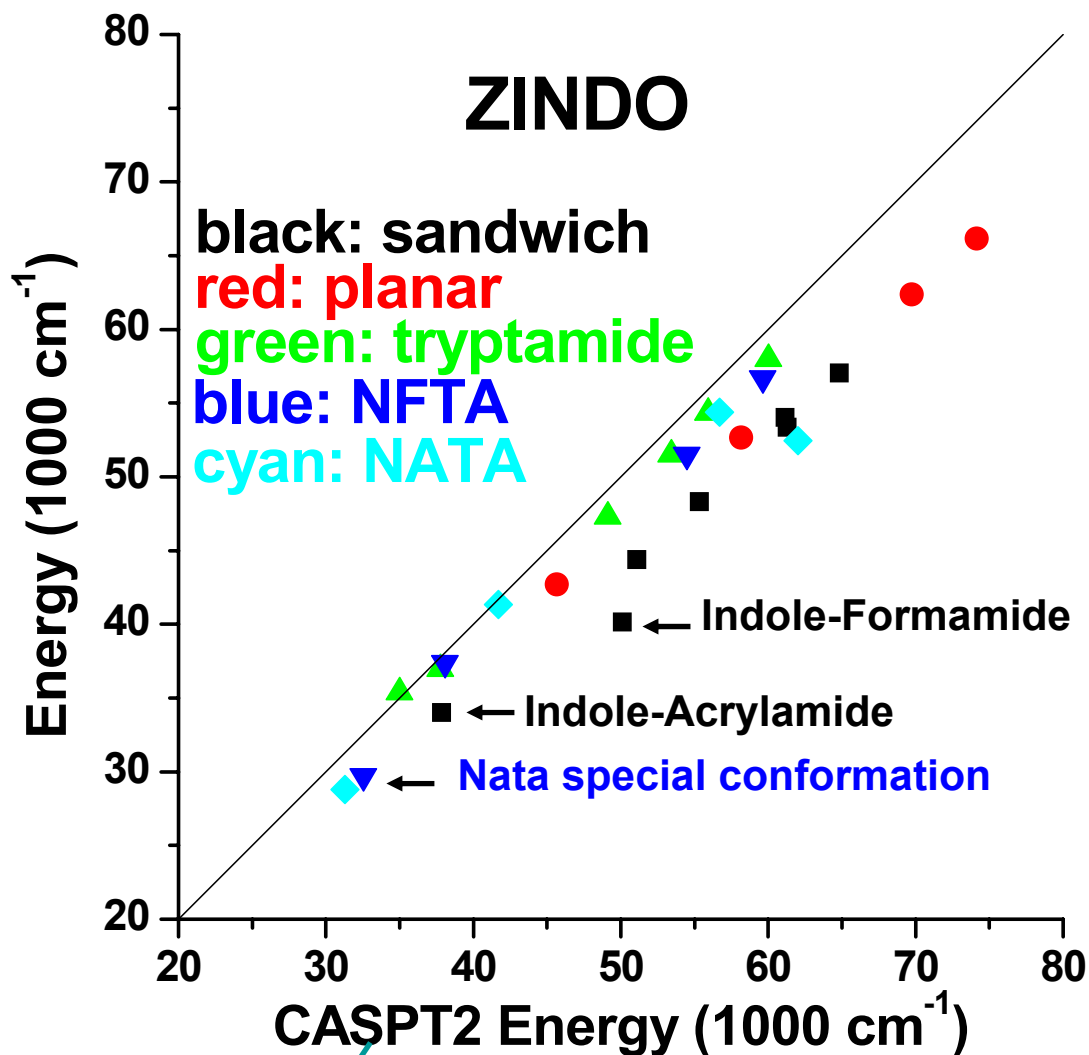
Zerner's INDO/S-CIS (ZINDO): Excellent track record for pi-pi* states, but UNKNOWN performance for indole → amide CT state.

We therefore compared ZINDO with CASPT2 calculations of these CT state energies.

The results on the next slide show that ZINDO performs very well, whereas another inexpensive and popular method TDDFT fails for CT states such as these.

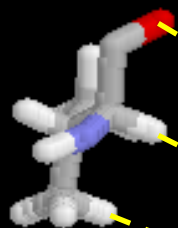
In the following slide “sandwich” and “planar” refer to indole—formamide stacked and in-plane non-covalent super molecules and Nata refers to N-acetyltryptophanamide.

Validating the semiempirical quantum chemistry



“Gold standard” for excited states. We acknowledge Andrzej Sobolewski for help with the MOLCAS program.

Putting the environment into the ZINDO calculations

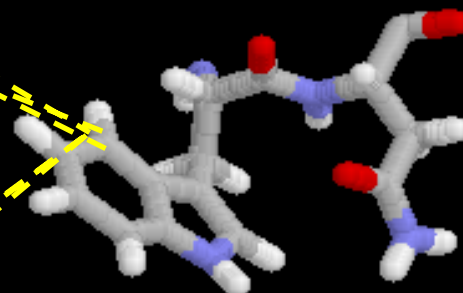


$r_{\alpha k}$

Electric potential at atom α of Trp

$$V_{\alpha} = \sum_k q_k / r_{\alpha k}$$

where sum is over **ALL atoms**,
protein and water.



potential at atom a = $V_{\alpha} = \sum_k q_k / r_{\alpha k}$ Volts

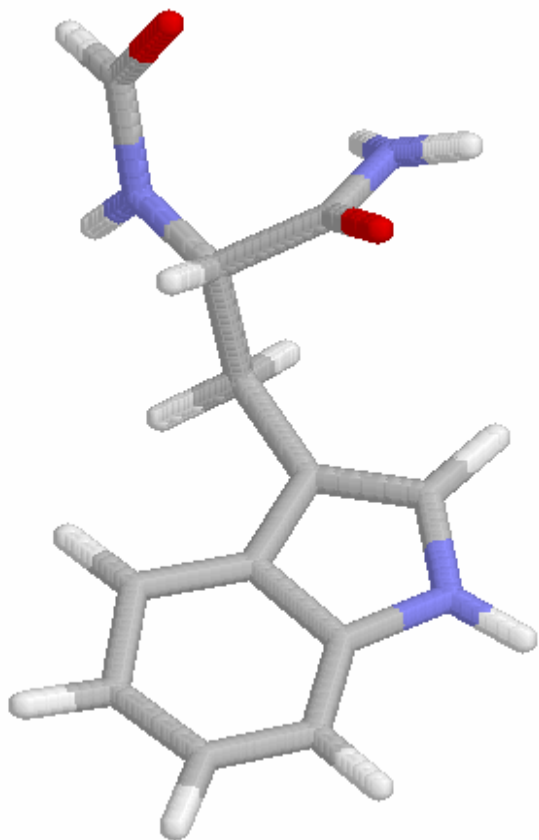
The potential for each atom in the quantum mechanical part is added to the Hamiltonian of the INDO/S (Zindo) program.

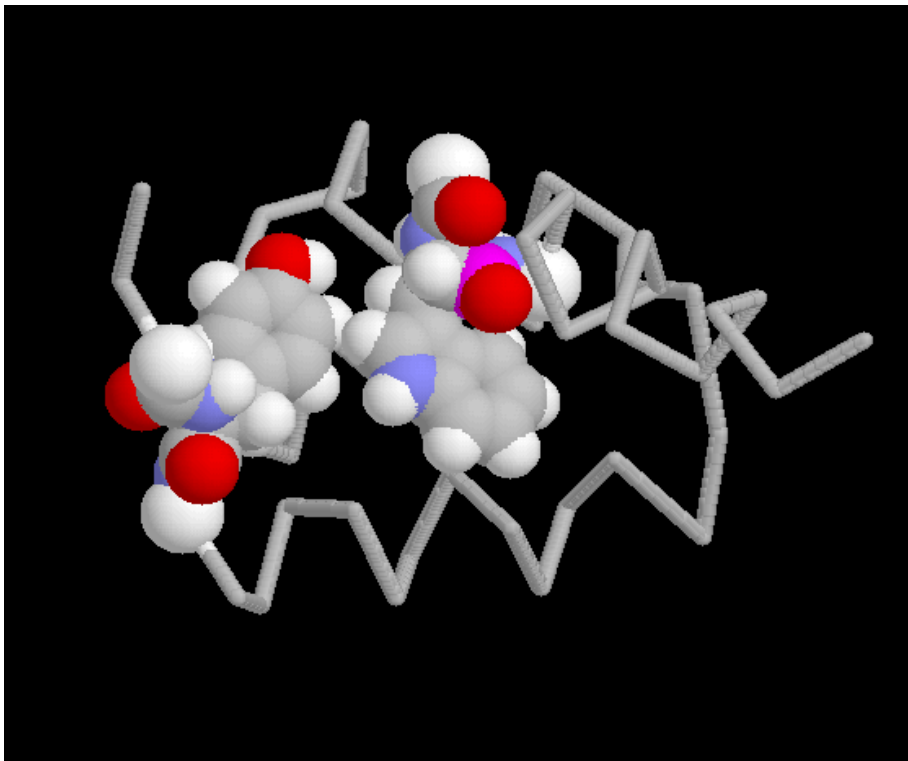
The transition energies are thus changed by $\sum_{\alpha} V_{\alpha} \Delta\rho_{\alpha}$

where, $\Delta\rho_{\alpha}$ = change of charge on atom α

This gives an energy shift in eV

(1 eV = 8066 cm⁻¹)





Here is an example of the Trp48 - Tyr8 pair of UBX. The two molecules, though not covalently bonded, are treated as a single molecule.

Some of the excited states are purely charge-transfer.

Where does the electron go??? **My starting point** was that perhaps the electron escapes the Trp to an area of especially positive electrical potential, *anywhere* in the protein.

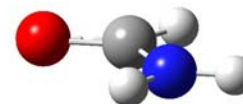
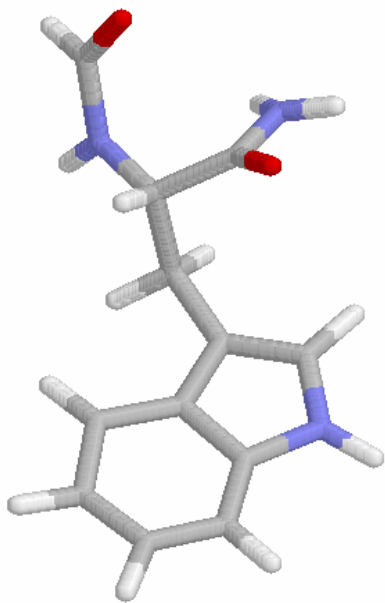
But, the magenta C atom on Trp is the primary electron acceptor in almost all cases.

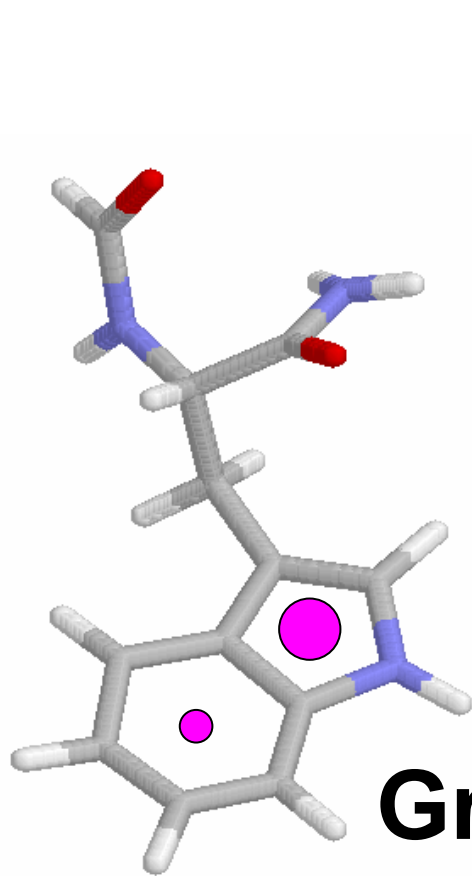
The CT states are just part of the excited state manifold of the pair.

Electron transfer from the 1L_a state of Trp is equivalent to internal conversion between two excited states.

This requires that, through environmental fluctuations, the two states have the **same energy--the original Marcus idea.**

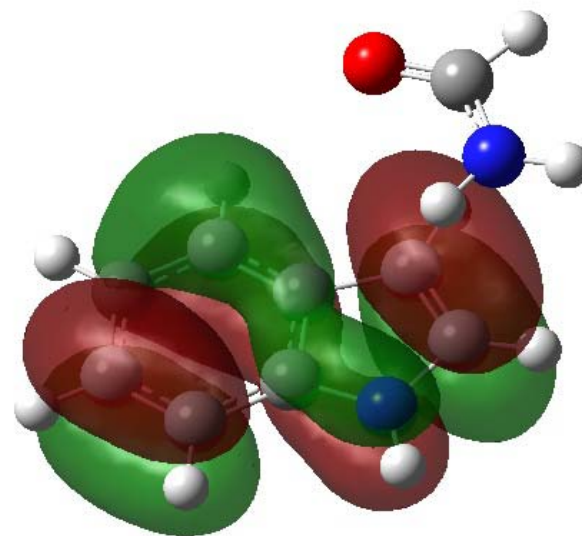
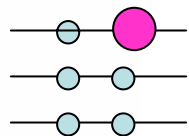
An informative model: Instead of the covalently bonded amide system, we first did calculations on a simpler system: Indole - Formamide coplanar sandwich.





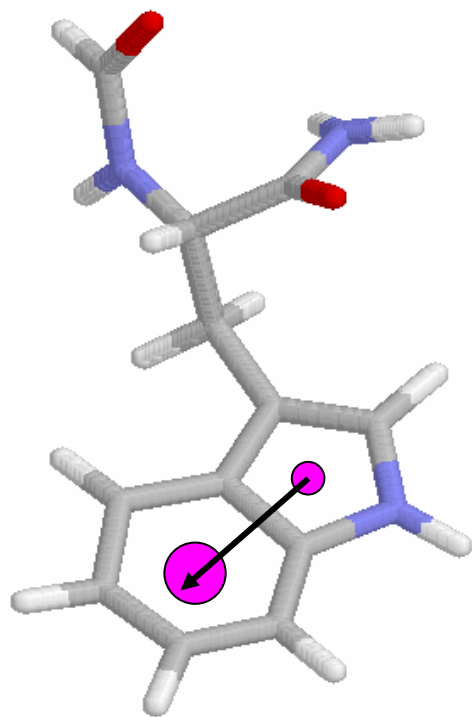
— Amide

— Ring



Ground State

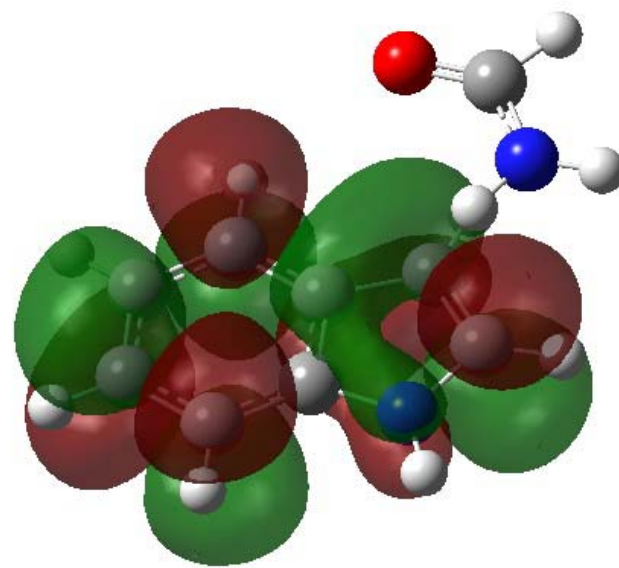
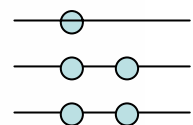
**Highest Occupied Molecular
Orbital**



Electron transfer
direction

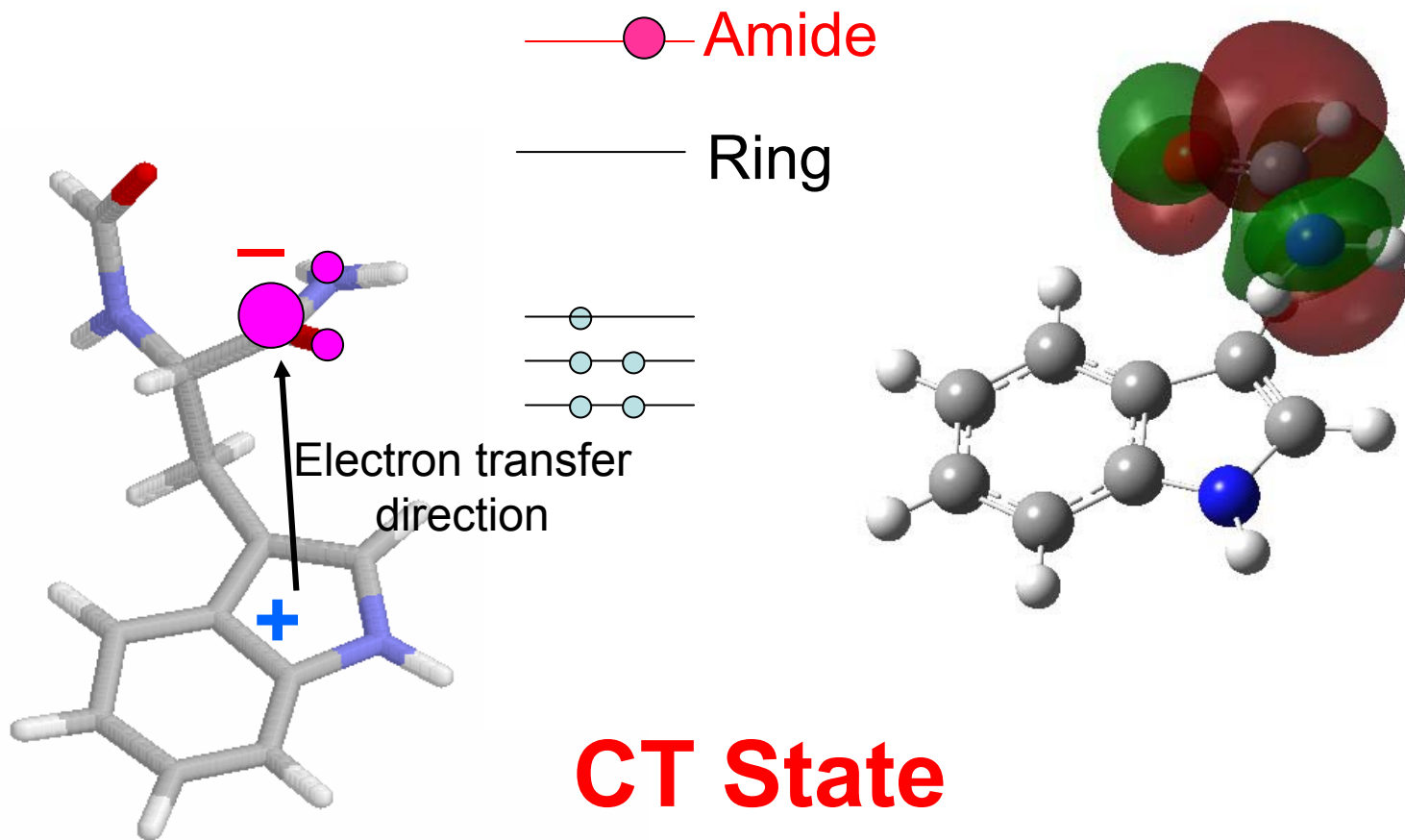
— Amide

— Ring



L_a State (fluorescing state)

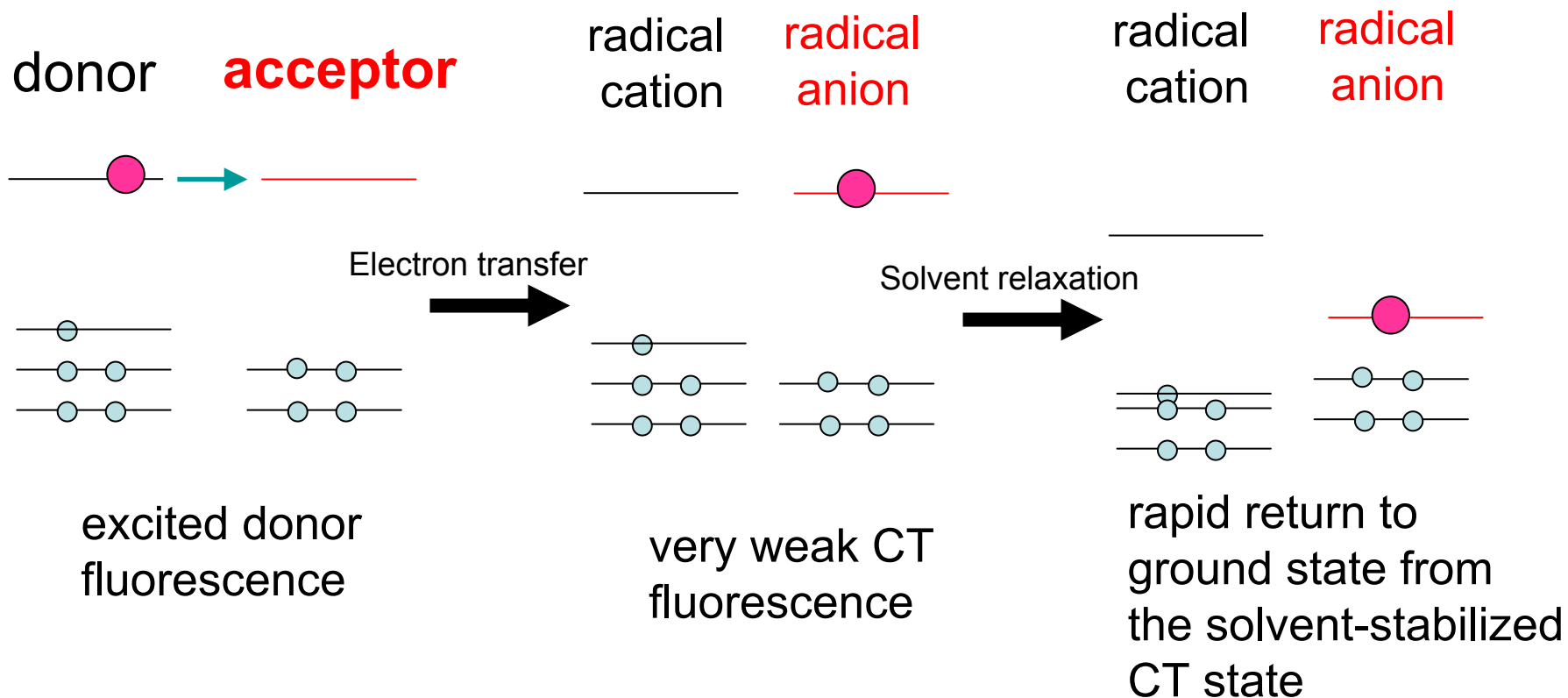
**Lowest Unoccupied Molecular
Orbital (electron excited)**



**Lowest Unoccupied Molecular
Orbital of Amide (π^*)
(electron transferred)**

Why donor fluorescence is quenched by electron transfer

(This is a reasonable model if there are covalently bound methylenes between the amide and ring.)



QM-MM Simulations

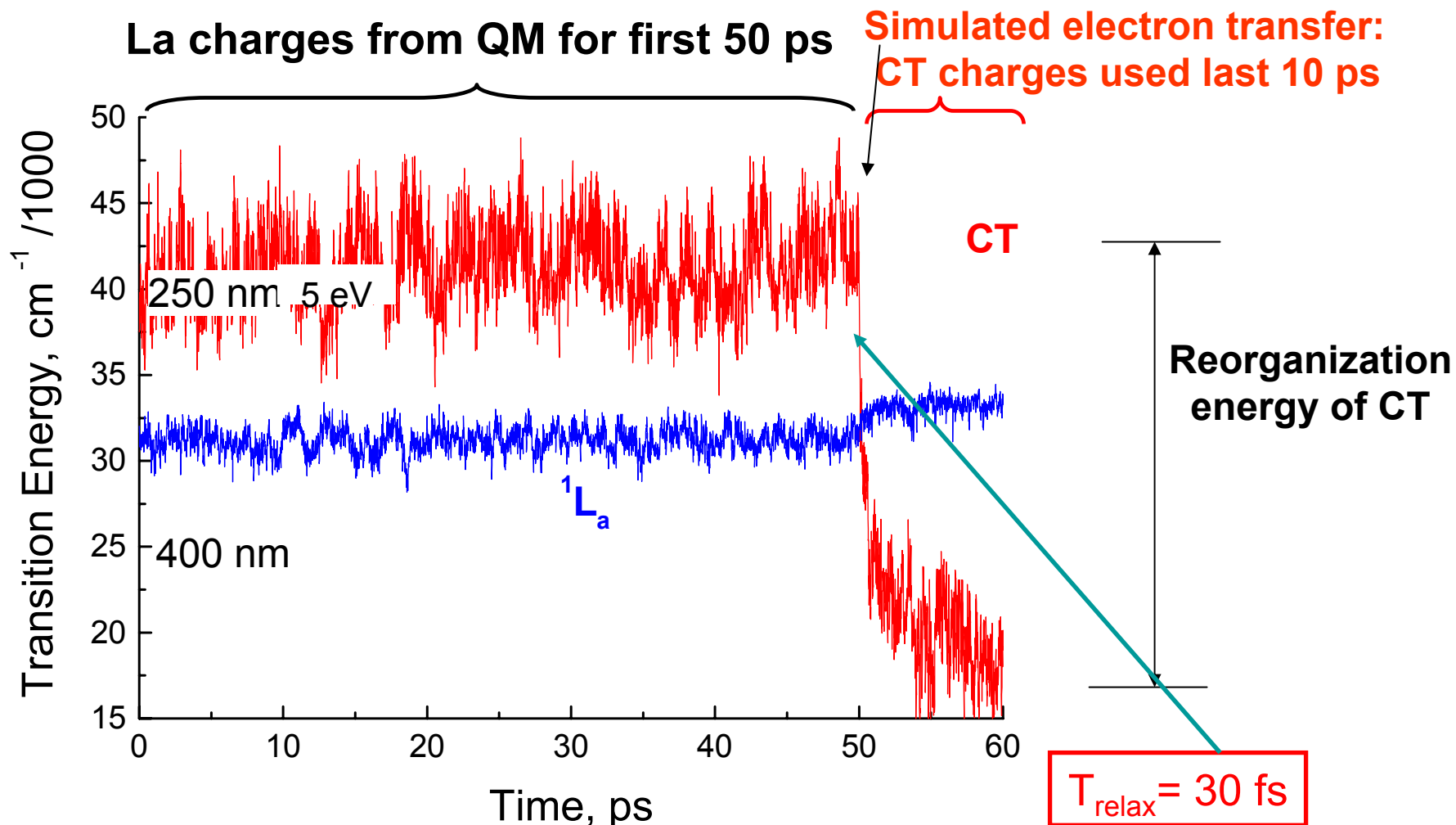
In the simulations of CT and 1La energies relative to the ground state shown in the following figures, the CT state energies are shown in red and 1La in blue. (1 eV = 8066 cm⁻¹)

For the first 50 ps, the QM computed 1La charges are fed back to the MM every 10 fs. Arbitrarily, at 50 ps a simulated electron transfer is executed by feeding the lowest CT state charges back to the MM every 10 fs for the remainder of the trajectory.

The transition energies are vertical at the CT geometry, as determined by optimized geometries of the indole radical cation and the amide radical anion.

QM-MM Simulations, (using *CT geometry*)

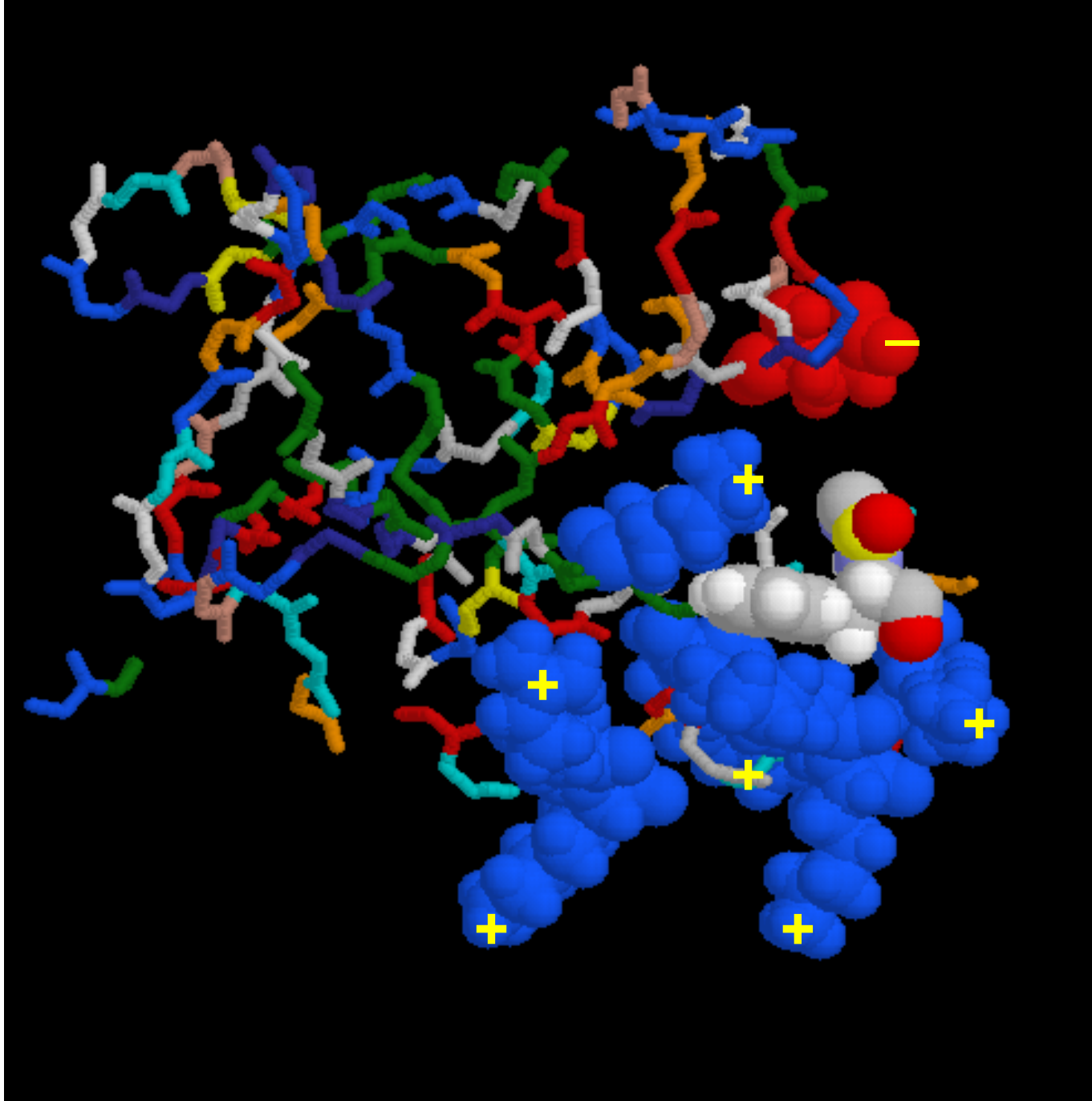
Staph. Nuclease: HIGH Quantum Yield = 0.29



The large energy gap seen between the fluorescing state (**blue**) and the CT state (**red**) in the preceding figure will be seen to translate into a very low quenching rate due to electron transfer.

For this case the primary reason for the large energy appears to be the uneven distribution of positively charge residues near the Trp ring and negative charges nearer to the amide. This adds a large increase in energy for transferring the electron from the ring to the amide.

The major contributing residues are shown in the following figure as spacefilling, with positive residues (Lys and Arg) **blue** and negative (Glu and Asp) as **red**. The Trp is spacefilling and CPK colored. The C for which the electron transfer gives the lowest energy 80% of the time is shown in yellow



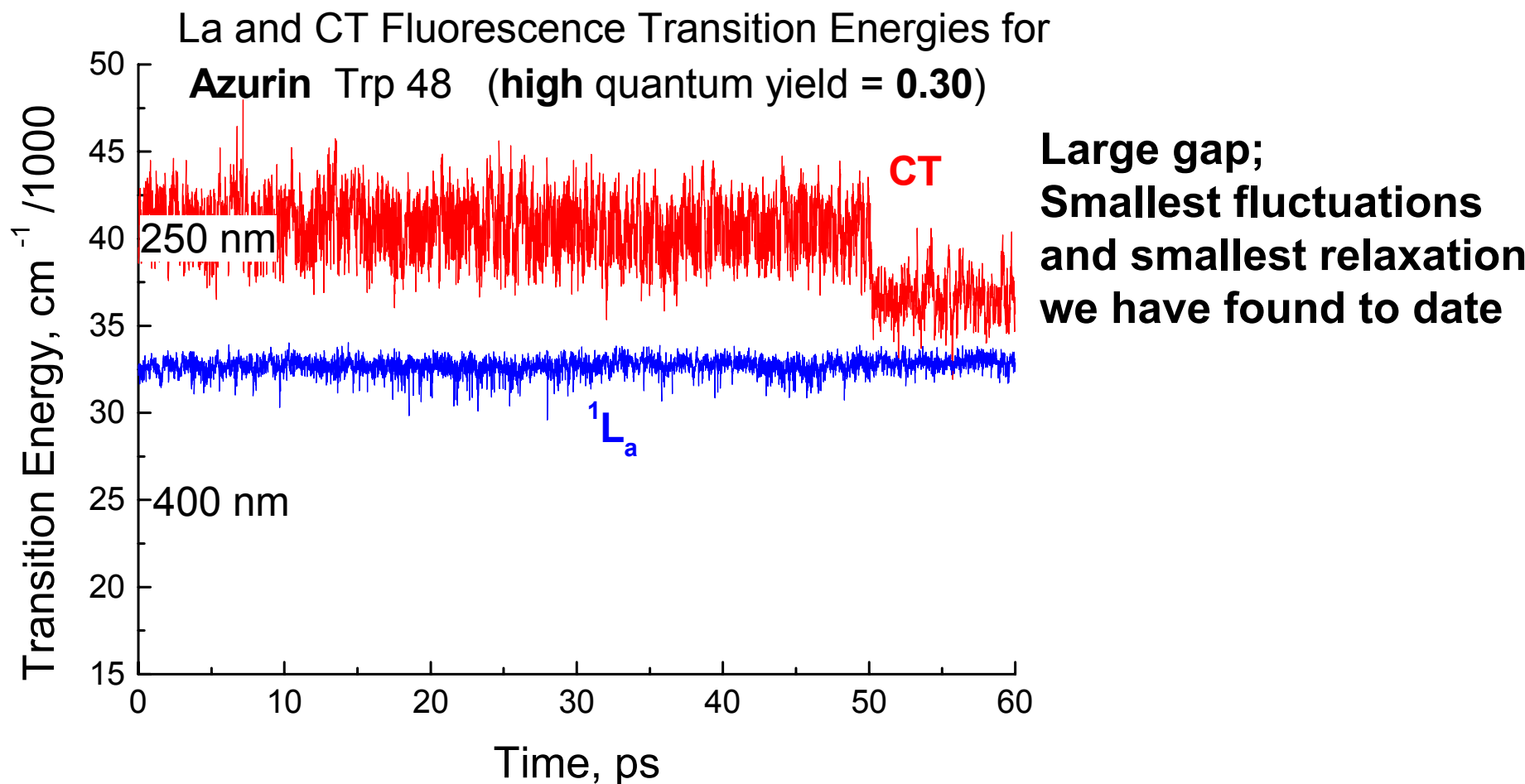
Largest contributors* to the CT-La energy gap (at a typical configuration)

	Group	Ave. Distance	Shift (cm-1)
GLU	57	14.52	1376
ARG	126	14.57	1630
LYS	110	8.05	1822
LYS	136	7.78	1927
ARG	105	11.75	2016
LYS	134	11.41	2070
LYS	133	6.48	<u>7954</u>

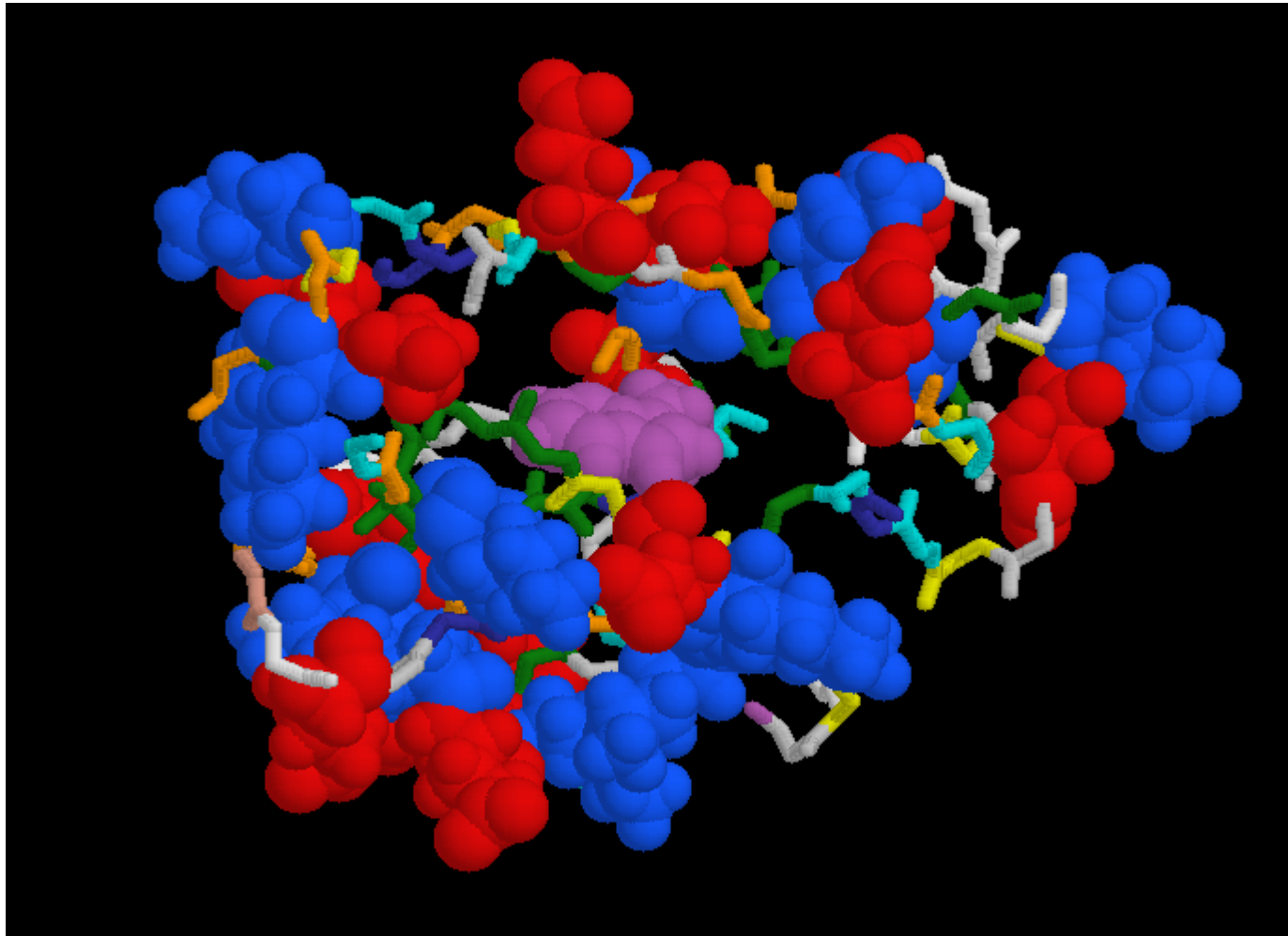
Sum = 18,800 cm⁻¹ = 2.3 eV

* Actually, sum of all positive contributions from the protein is about +5 eV and the sum of all stabilizing contributions is about -3.4 eV. The surrounding water is highly polarized by the surface charges on the protein. The water contributes net stabilization of about -1.7 eV. It is necessary to include all point charges in the simulation.

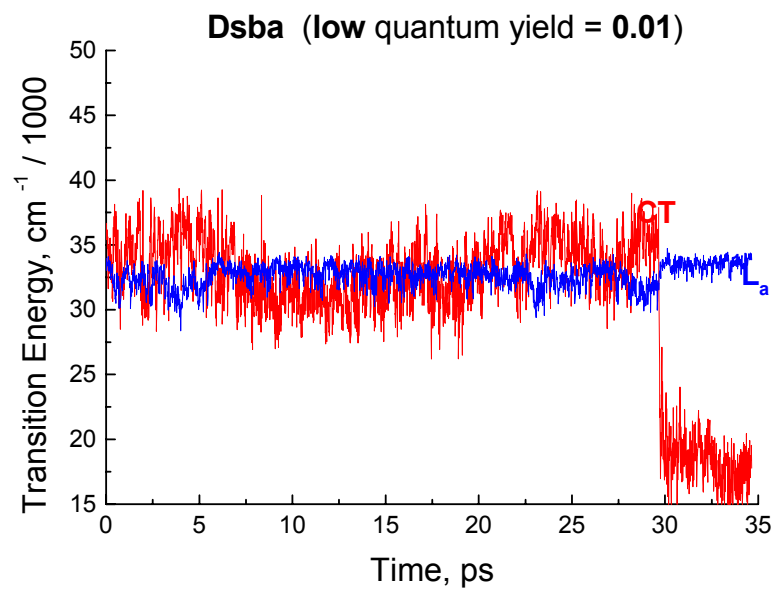
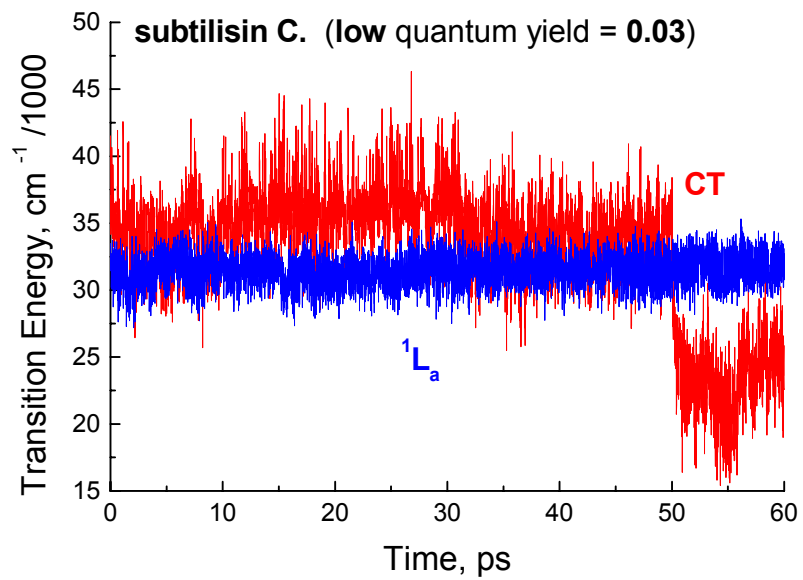
A second high quantum yield example: Trp48 of azurin is in a completely hydrophobic pocket, 10 Angstroms from nearest water.



For azurin, shown below, charged groups and water are all distant from Trp 48 and the distribution is more uniform than for staph nuclease.



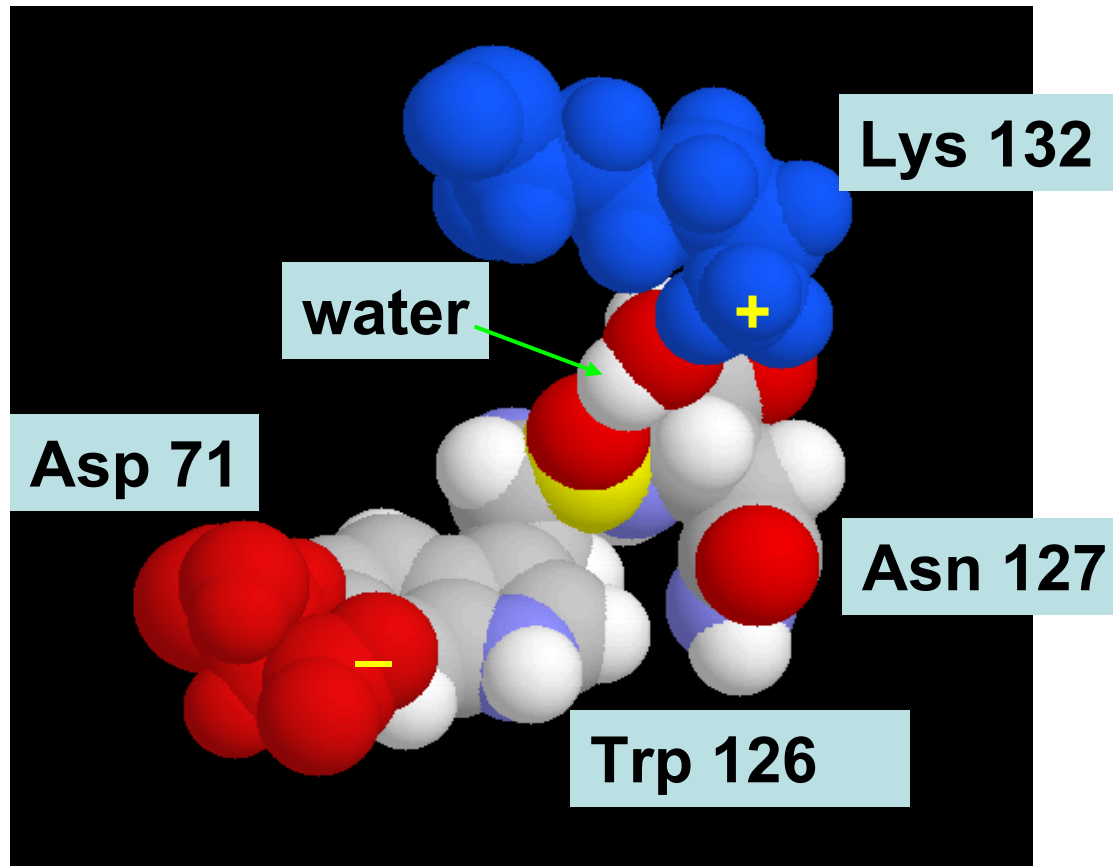
NEXT: Low Quantum Yield Cases

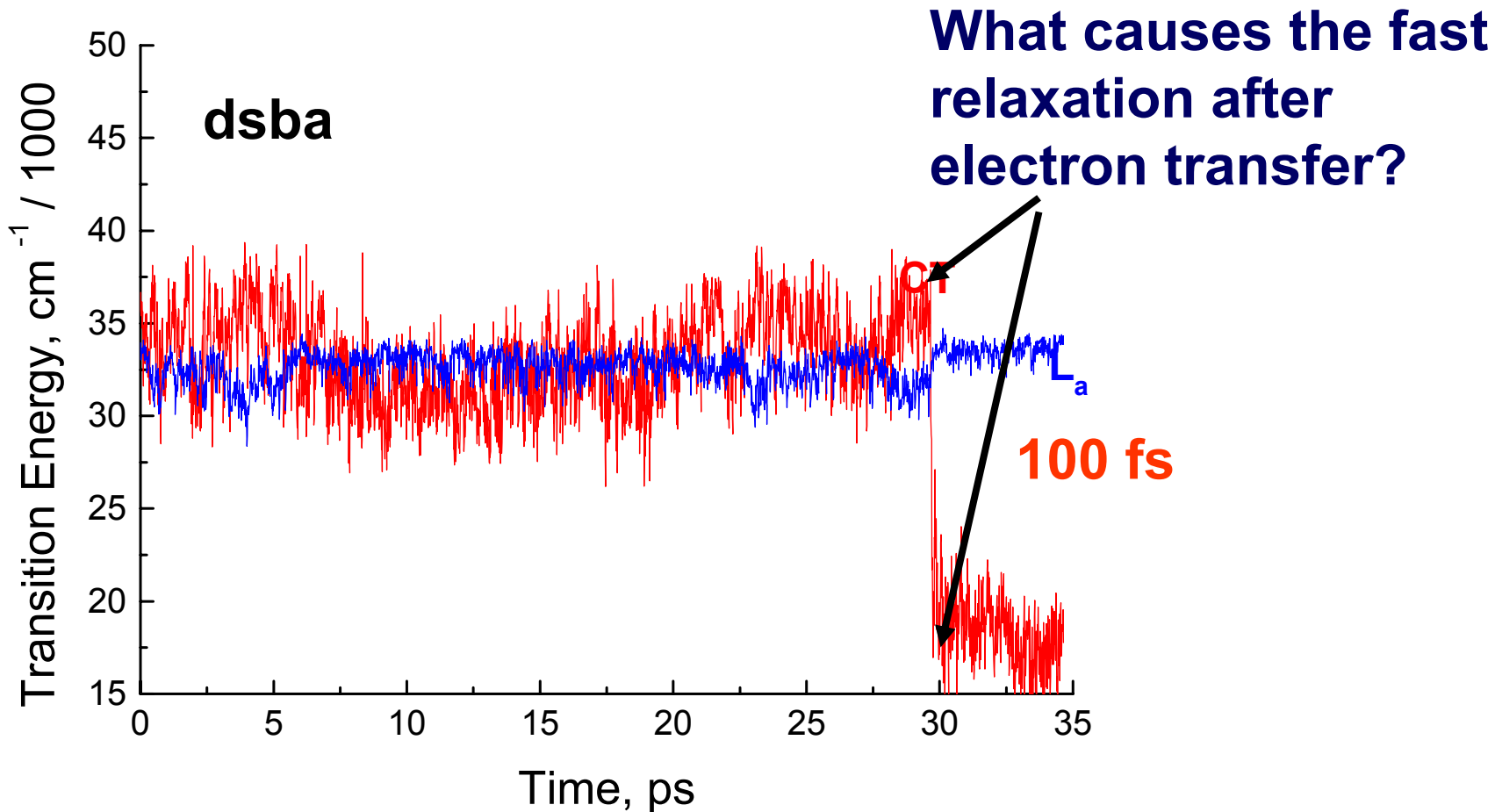


DsbA: small gap, low yield

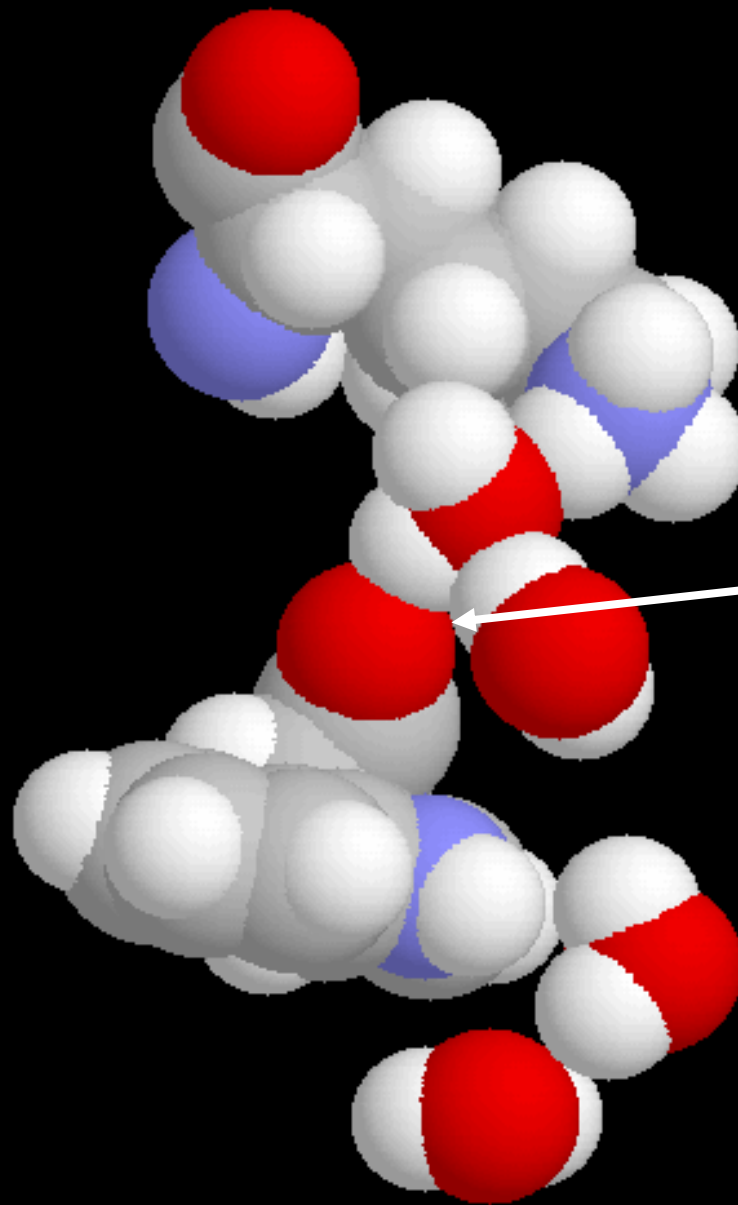
asn127, lys132 and asp71, and a water
stabilize by about 2 eV (16000 cm^{-1})

negative at ring **and positive** at the carbonyl





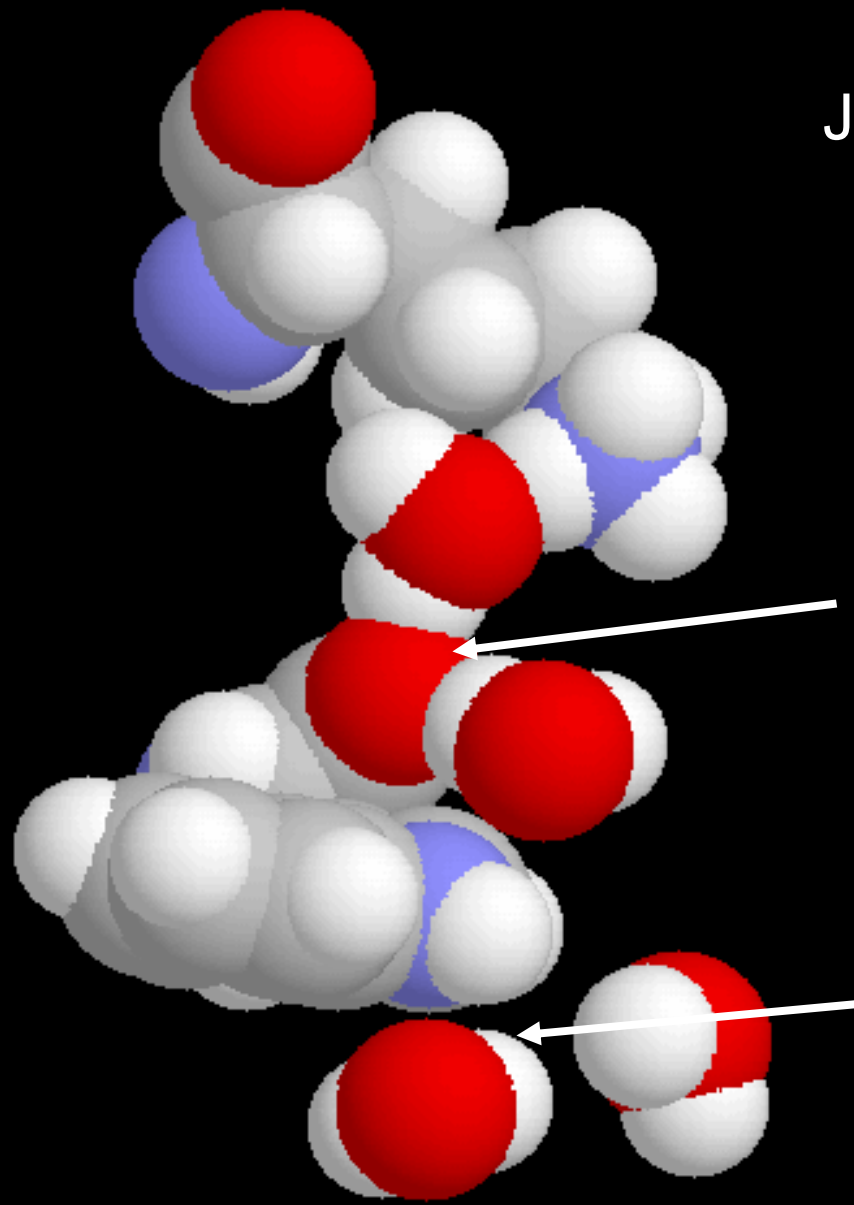
Most of the 2 eV relaxation of the CT state following simulated electron transfer comes from surprisingly subtle motion of nearby charge and polar groups, as see in the next two figures:



Just **before**
electron
transfer

only **one**
hbond to
carbonyl

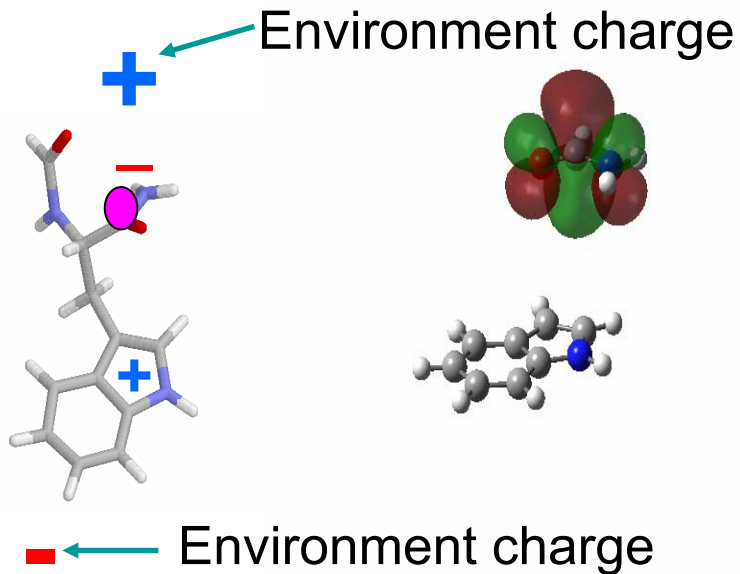




Just **AFTER**
electron
transfer

Now, **TWO**
hbonds to
carbonyl

Waters now
face oxygen
toward the
positive ring

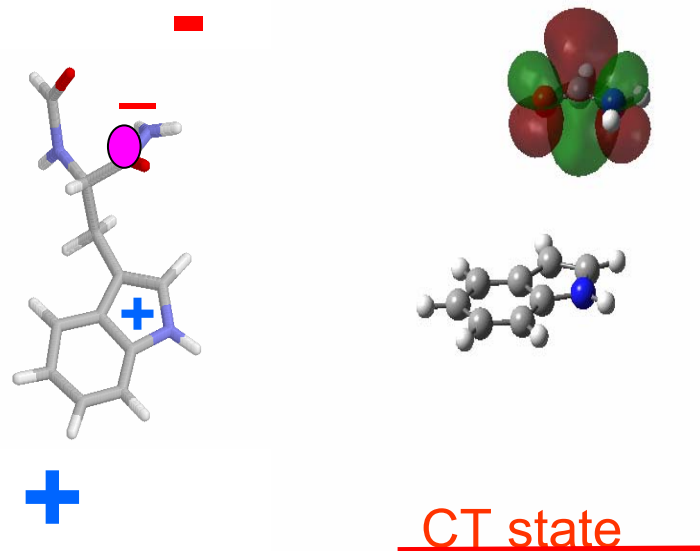


Electron transfer is possible only if the two states have equal energy.

--R. Marcus

If environment charges stabilize the CT state :

LOW Quantum Yield



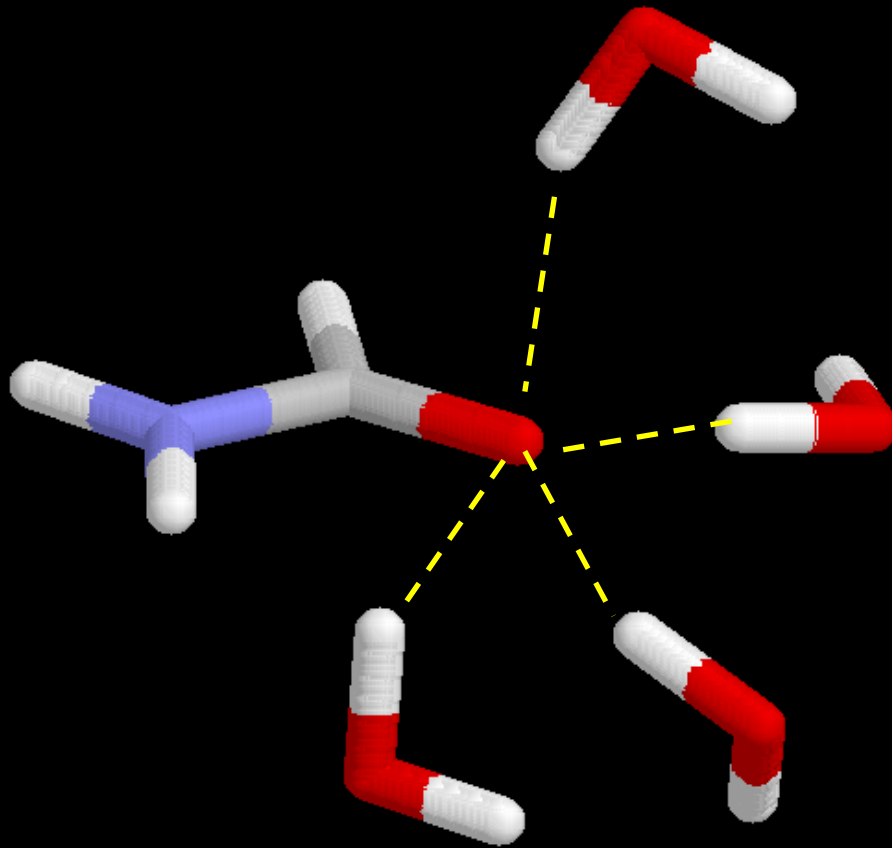
Electron transfer **not possible** if the two states never have equal energy.

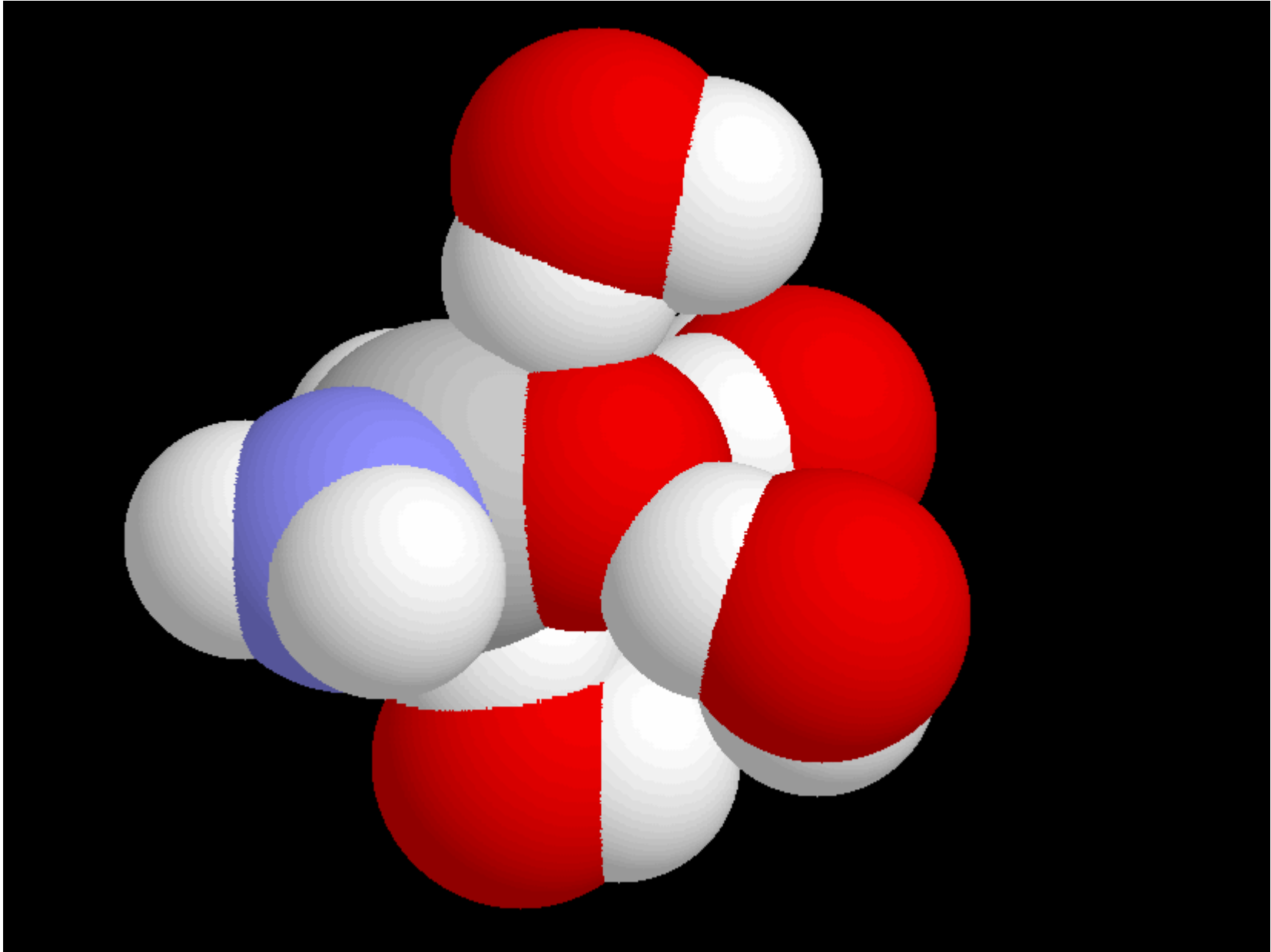
If environment charges **destabilize** the CT state :

HIGH Quantum Yield

For high exposure to water:

Simulations say the solvent stabilized CT state has
FOUR H-bonds to carbonyl near the transferred electron

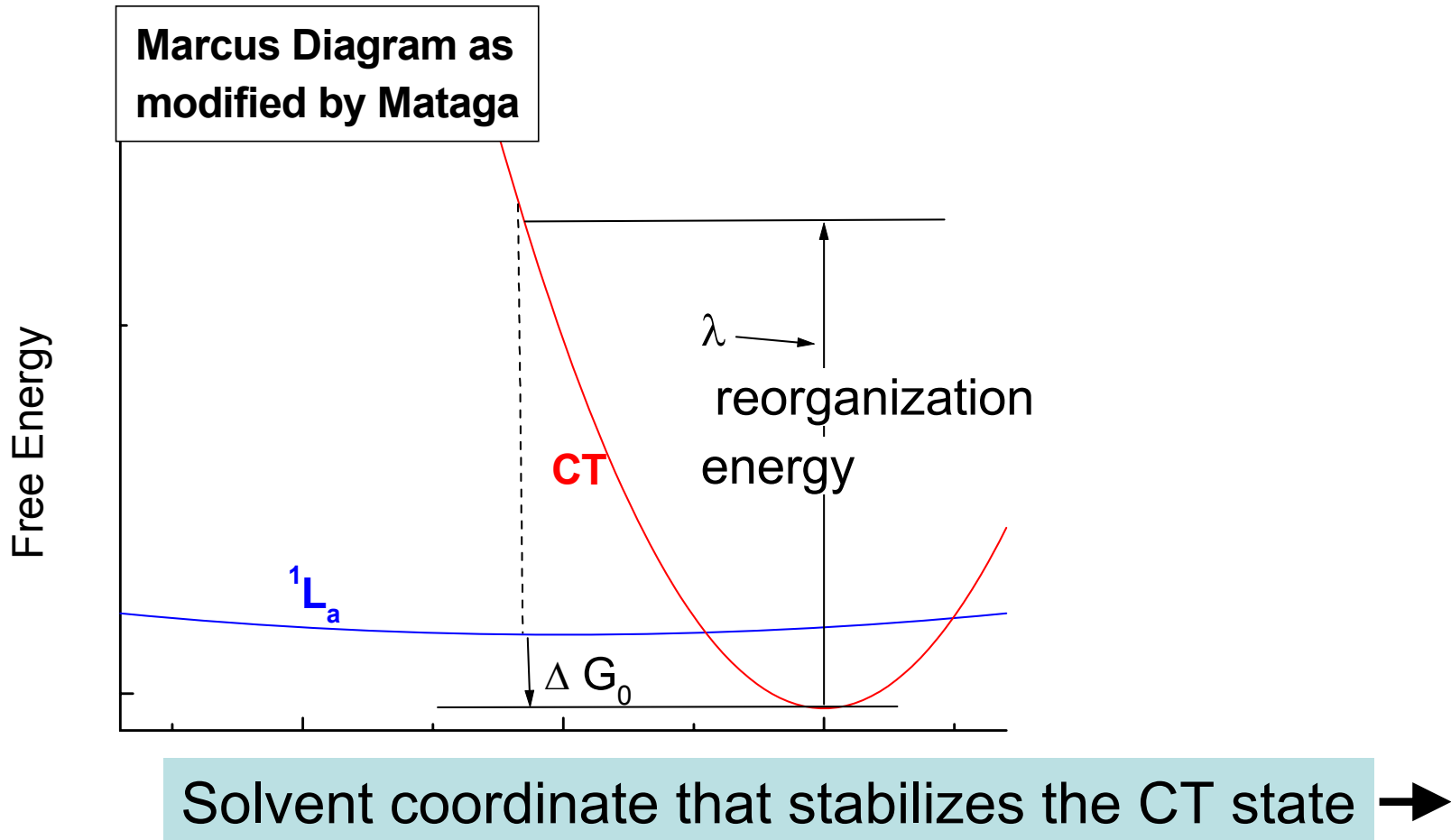




Towards Quantitative Prediction

$$k_{\text{ET}} = \left(\frac{4\pi^2}{h} \right) V^2 F \left(4\pi\lambda k_{\text{B}}T \right)^{-1/2} \exp \left(- \frac{(\Delta G_0 + \lambda)^2}{4\lambda k_{\text{B}}T} \right) \quad (2)$$

Where V = electronic interaction matrix element, F = effective Franck-Condon factor and ΔG and λ are defined below



The Fermi Golden Rule expression for the electron transfer rate constant is

$$k_{ET} = \frac{2\pi}{\hbar} \langle V^2 \rangle \rho(\Delta E_{00})$$

V = **electronic matrix element coupling the CT and La states**

ΔE_{00} = **energy difference of vibrational zero points of CT and 1L_a**

$\rho(\Delta E_{00})$ = **density of final vibrational states for a given ΔE_{00}**

$$\rho(\Delta E_{00}) = \int F_{D \rightarrow D^+}(\Delta E_{00}, E) F_{A \rightarrow A^-}(\Delta E_{00}, E) dE$$

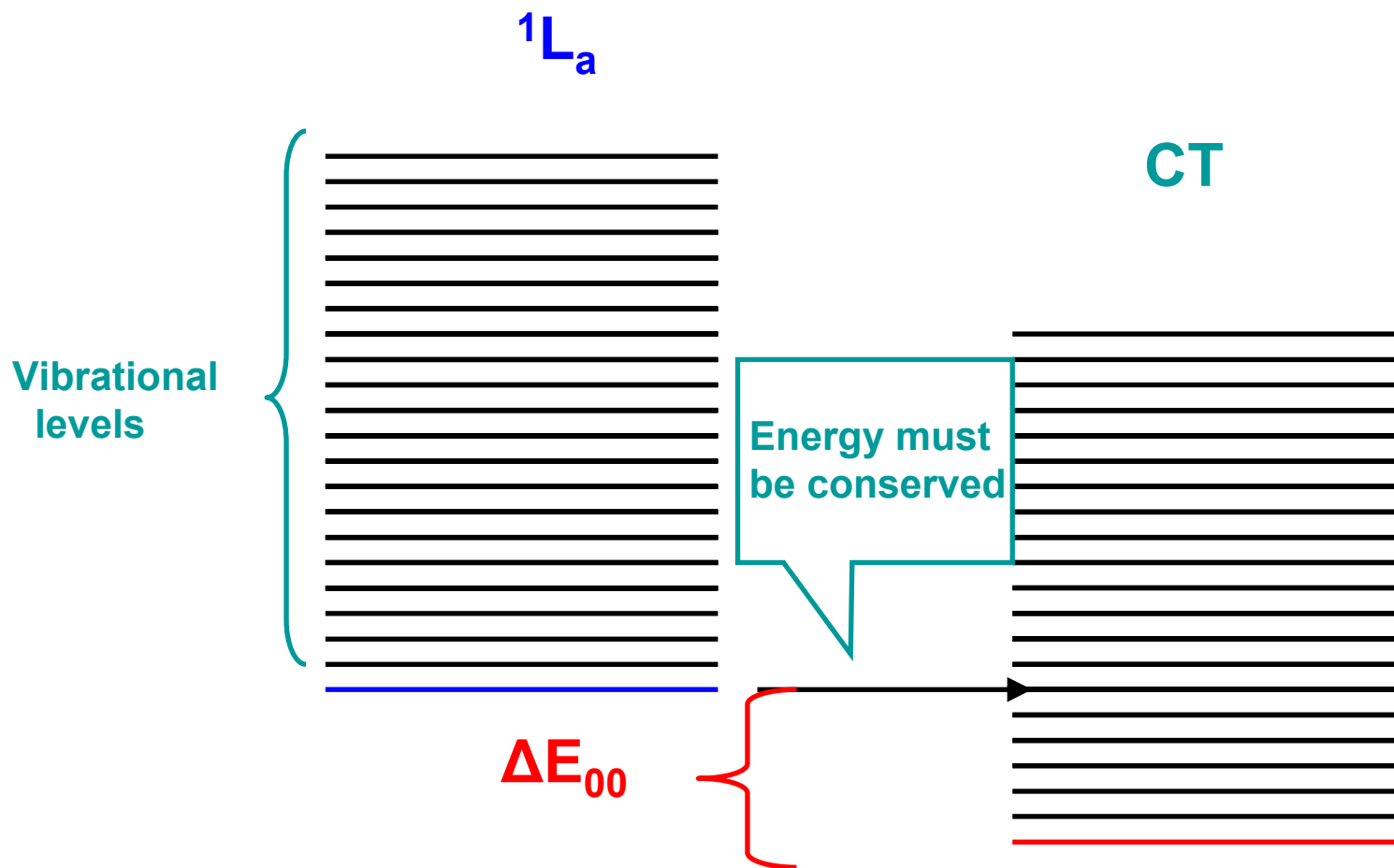
= **the overlap** of the photoelectron spectrum of the indole ring with that of the acceptor anion (**similar to FRET**)

--Hopfield, 1976

We assume that fluctuations in the environment modulate ΔE_{00} randomly and rapidly on the fluorescence lifetime scale, leading to a gaussian distribution for ΔE_{00} and an average ET rate constant given by,

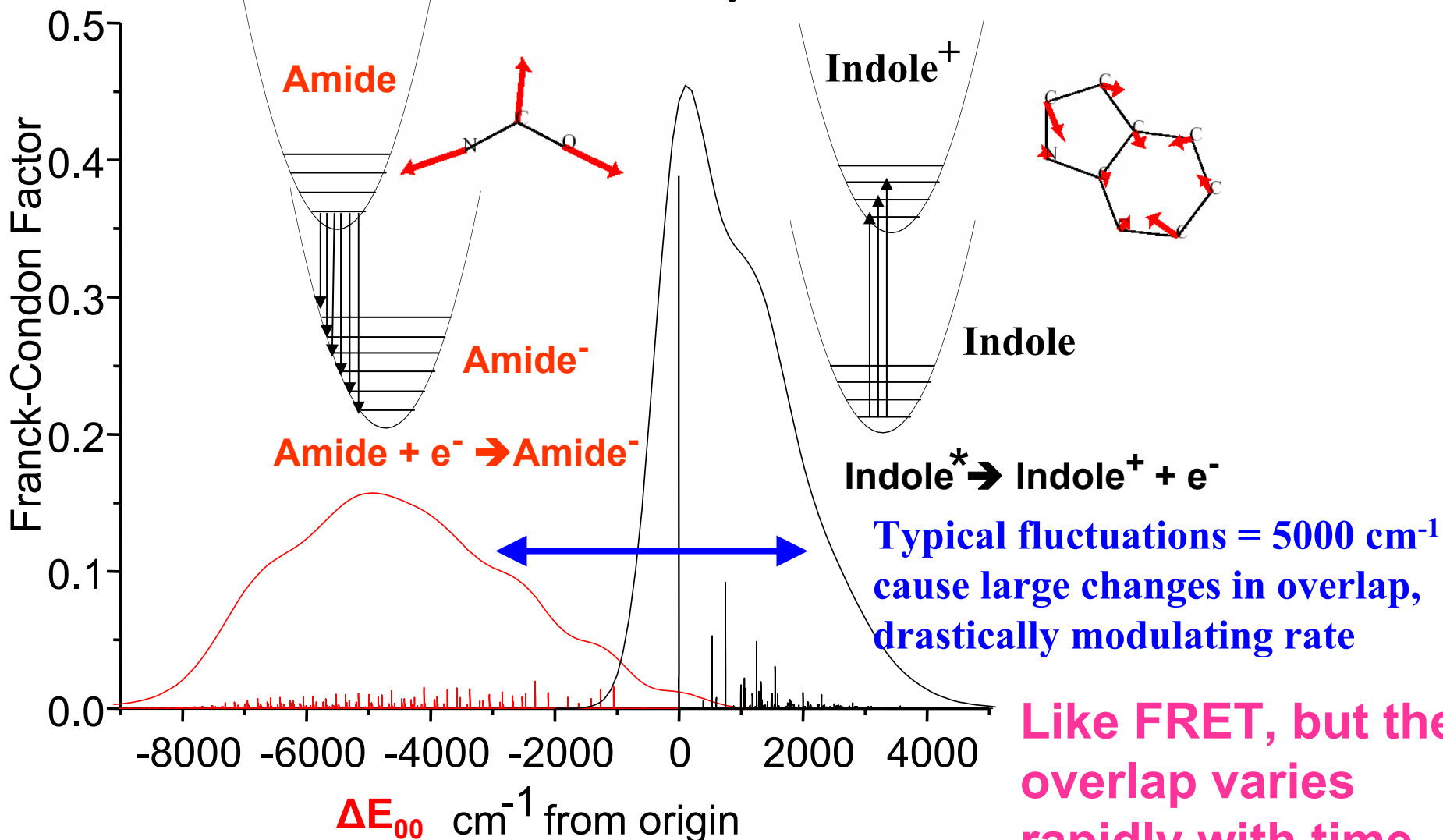
$$\langle k_{ET} \rangle = 4\pi^2 c \langle V^2 \rangle (2\pi\sigma^2)^{-\frac{1}{2}} \int \rho(\Delta E_{00}) e^{-\frac{1}{2} \left(\frac{\Delta E_{00} - \overline{\Delta E_{00}}}{\sigma} \right)^2} d\Delta E_{00}$$

For molecules, there are vibrational states associated with every electronic state. CT transitions will usually be to a vibrationally excited level of the CT state



Bondlength changes and Franck-Condon factors for electron transfer

Geom diff. from CIS or HF/3-21G for indole-formamide dimer. Normal modes and frequencies from B3LYP/6-311+g** on ground states of indole and formamide. Lines are broadened by 1000 cm^{-1}



Ad hoc Semi-quantitative prediction

At present, we obtain $\langle \Delta E_{00} \rangle$ and the standard deviation from the QM-MM trajectories. Because the distance to nearest amides is difficult to define, and is by any measure similar in all cases, we have taken the electronic coupling to be a constant.

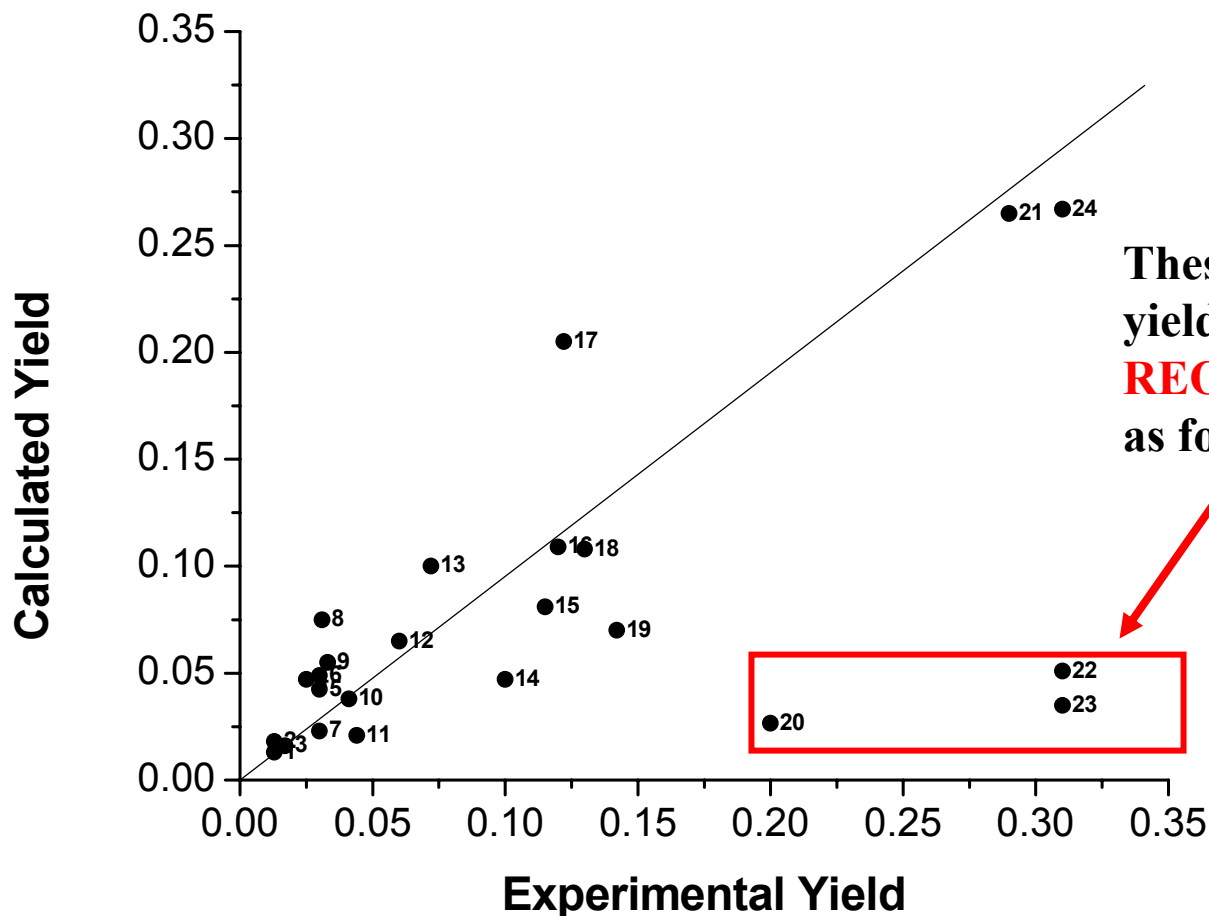
In addition, an empirical constant D is added to the computed $\langle \Delta E_{00} \rangle$, to correct for uncertainties introduced by choice of geometry, semiempirical energies, electrical potentials, electronic polarization, etc.

The following figure plots computed vs. observed quantum yields using $V = 10 \text{ cm}^{-1}$ and $D = -4000 \text{ cm}^{-1}$

Quantum yield = $k_{\text{rad}} / (k_{\text{rad}} + k_{\text{nr}} + k_{\text{ET}})$, where electron transfer rate constant, k_{ET} is given by:

$$\langle k_{\text{ET}} \rangle = 4\pi^2 c \langle V^2 \rangle (2\pi\sigma^2)^{-\frac{1}{2}} \int \rho(\Delta E_{00}) e^{-\frac{1}{2} \left(\frac{\Delta E_{00} - \overline{\Delta E_{00}}}{\sigma} \right)^2} d\Delta E_{00}$$

Empirical fit by setting ΔE_{00} = Diff. of CT and 1L_a transition energies - 4000 cm^{-1} and electronic coupling $V = 10 \text{ cm}^{-1}$ for all proteins



These misfits with high quantum yield have in common a **SMALL REORGANIZATION ENERGY**, as for RnaseT1 shown below.

Small reorganization energy is apparently an indicator of high quantum yield

Proteins in previous figure coded to numbers

TABLE 2: Experimental and Calculated Fluorescence Quantum Yields for 24 Trps in 17 Proteins

expt.	calc. ^a	num	run/abbreviation	description ^b	ref ^c
0.013	0.003	1	lyd158-asn2	T4 lysozyme W158-asn2 1lyd	111
0.013	0.005	2	dsb126	dsBa W126 1dsb	90
0.017	0.004	3	barn94-his18+	barnase W94-H18 pH5 1a2p	81
0.025	0.023	4	cpl	human cyclophilinA 2cpl	112
0.03	0.026	5	trpcage	TrpCage 112y (nmr structure 1&2)	7 ^d
0.03	0.035	6	d6o	fkf506 binding protein 1d6o	113 ^e
0.03	0.009	7	sbc	subtilisin C 1sbc	1 ^f
0.031	0.047	8	bpp	phospholipase A2 2bpp	1
0.033	0.018	9	nscp	NSCP W57 W4F W170F	28
0.041	0.042	10	dsbQ74A/N127A	dsba W126 Q74A, N127A mutated 1dsb	90
0.044	0.009	11	lyd138	T4 lysozyme W138 1lyd	111
0.06	0.047	12	lyd126	T4 lysozyme W126 1lyd	111
0.072	0.069	13	barn94	barnase W94 pH8 1a2p	81
0.1	0.030	14	ctx	cobra toxin 1ctx	1
0.115	0.058	15	mlt	melittin 2 mlt	1
0.12	0.080	16	gen	glucagon 1gen	1
0.122	0.225	17	barn71	barnase W71 his neutral 1a2p	81
0.13	0.121	18	b8r	parvalbumin 1b8r	1
0.142	0.048	19	barn35	barnase W35 his neutral 1a2p	81
0.2	0.014	20	dsb76	dsba W76 1dsb, 1fvk average result	90
0.29	0.275	21	stn	staph. nuclease 1stn	1
0.31	0.011	22	pfk	phosphofructokinase 6pfk	1
0.31	0.021	23	rnt	ribnuclease T1 9rnt	1
0.31	0.296	24	azb	apo-azurin W48 1azb	1

^a From eq 4 using $|V_{el}| = 10.0 \text{ cm}^{-1}$, $C_{00} = -4000 \text{ cm}^{-1}$. ^b Name, Trp sequence number or Trp-acceptor numbers, PDB code (all X-ray unless noted). ^c Reference for experimental quantum yield. ^d Estimated relative to NATA. ^e Upper limit based on the 12-fold increase upon denaturation. ^f Estimated from lifetimes.

High quantum yield

Feature Article

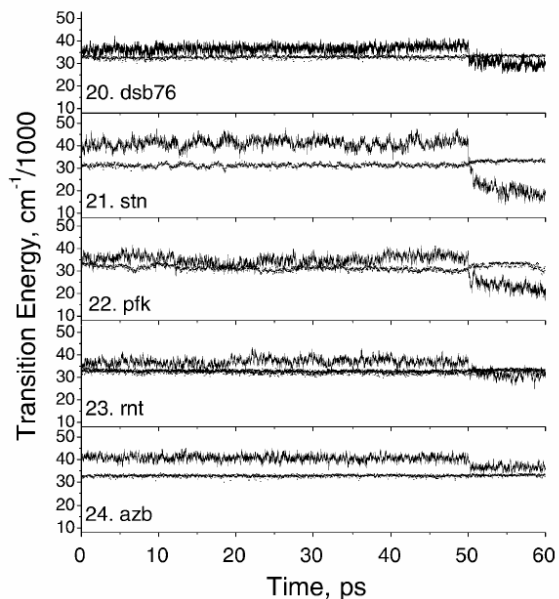


Figure 3. Vertical transition energy, $\Delta E/1000hc$ for the ground 1L_a state (dots) and the ground \rightarrow lowest Trp ring-amide charge transfer (CT) state transitions (line) during a 60 ps QM-MM trajectory for high quantum yield (quantum yields 0.2–0.3) tryptophans (Trps); bond lengths (but not angles and torsions) were held constant at values corresponding to the CT state (see Methods section). During the first 50 ps the charges on the Trp are those of the 1L_a state (scaled by 0.06 and 0.15) and are updated every 10 fs. During the last 10 ps the charges are those of the CT state. Note the much greater fluctuation amplitude for the CT state than for 1L_a .

Med

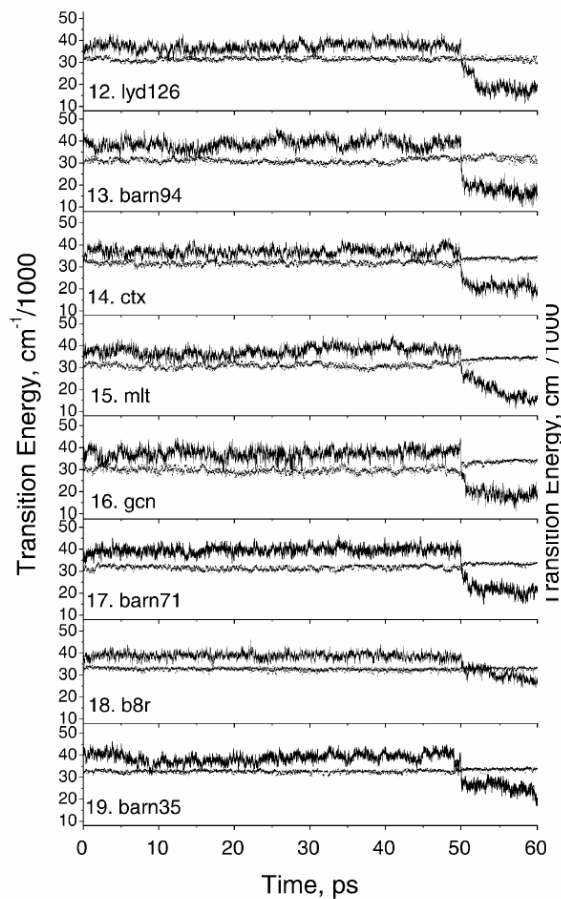


Figure 4. Same as Figure 3, except for Trps with quantum yields 0.06 and 0.15.

Low

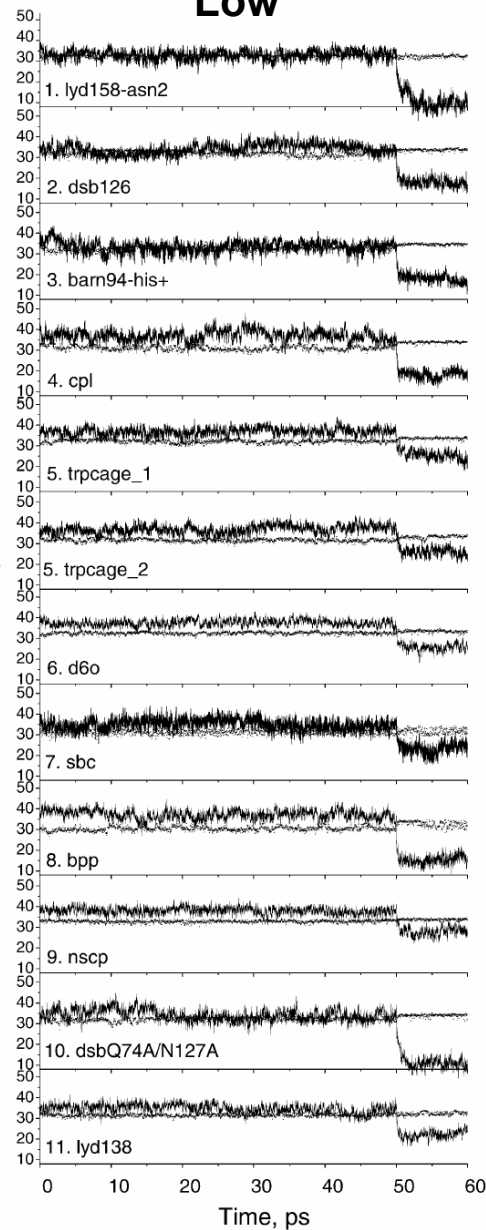
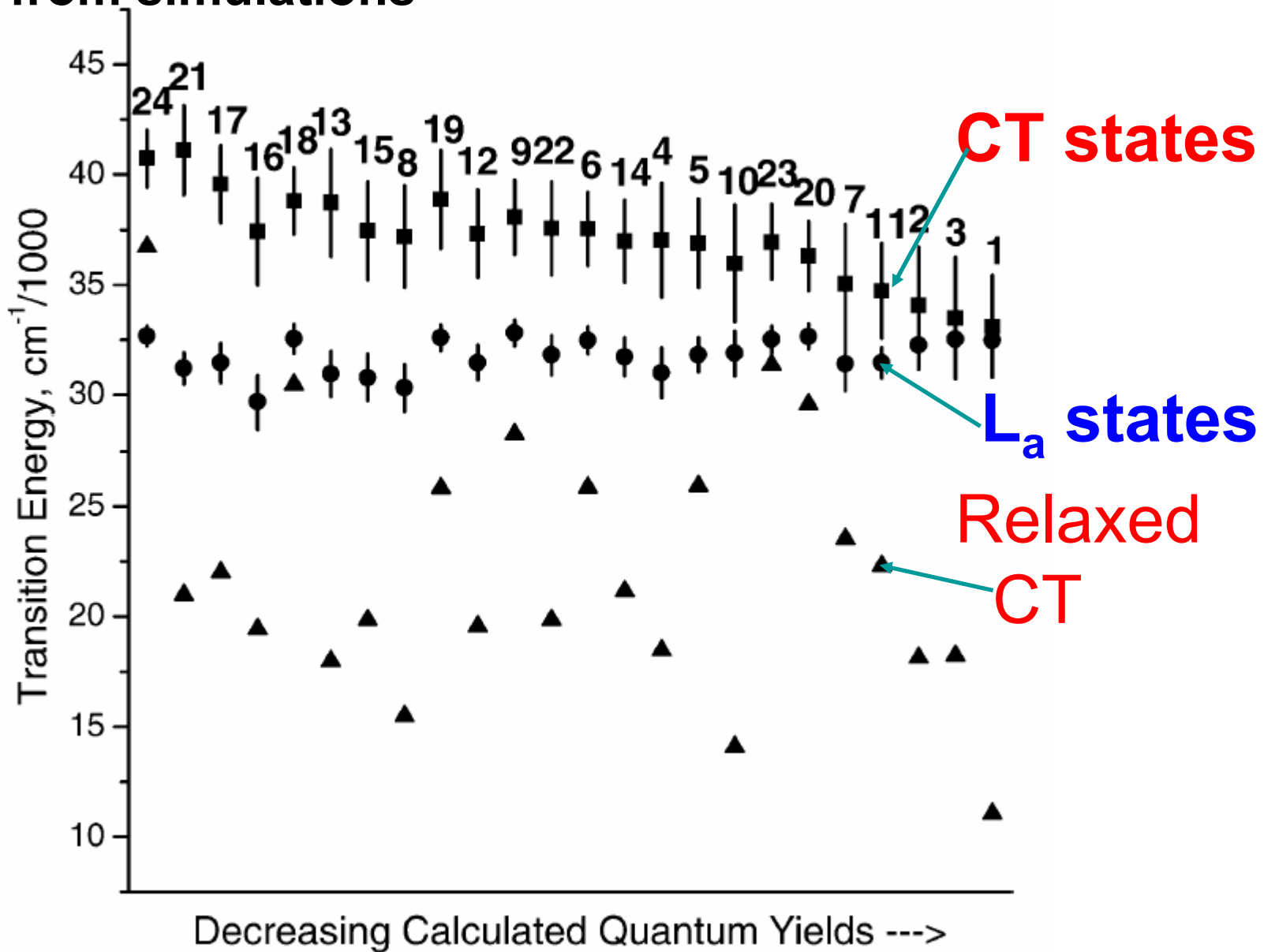


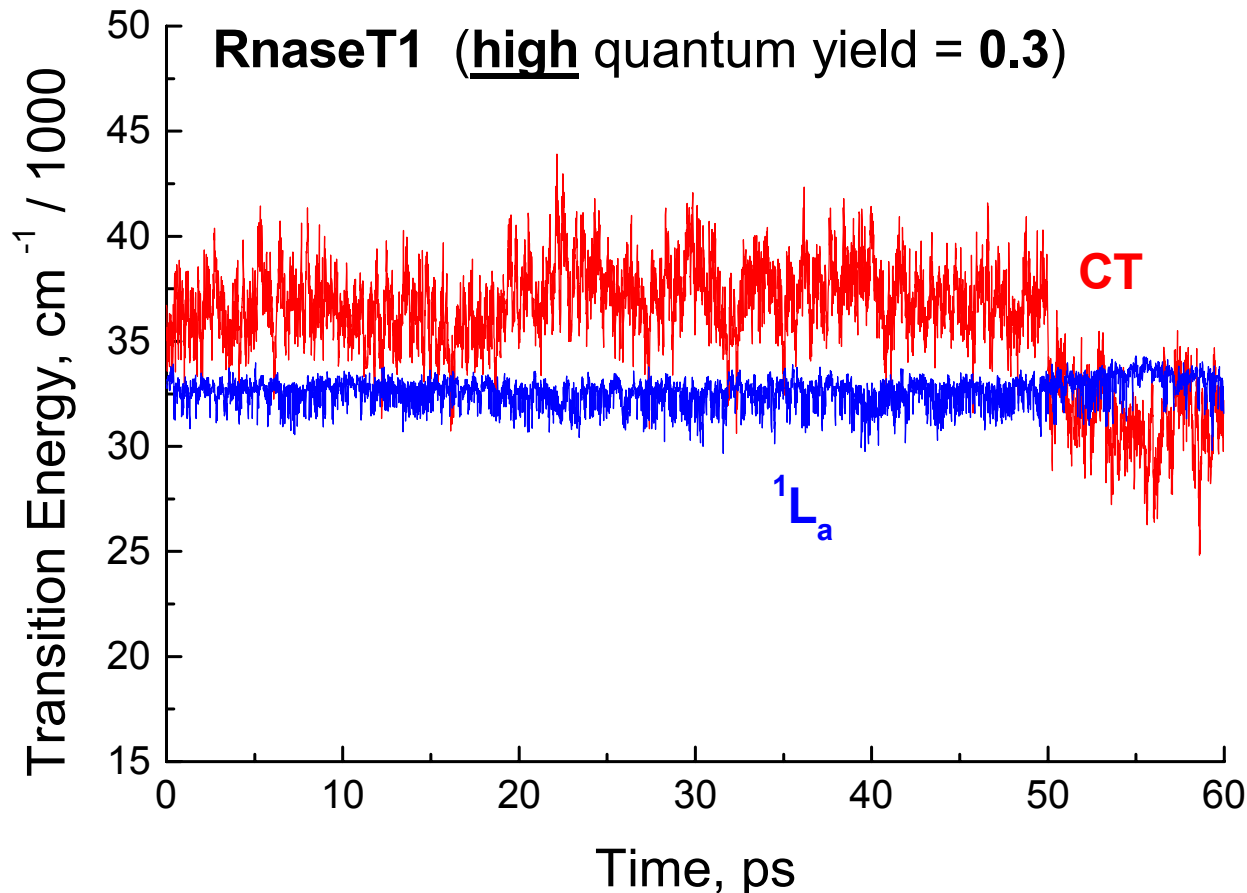
Figure 5. Same as Figure 3, except for Trps with quantum yields 0.01 and 0.05.

Energies, standard deviations (σ), and reorganization energies from simulations



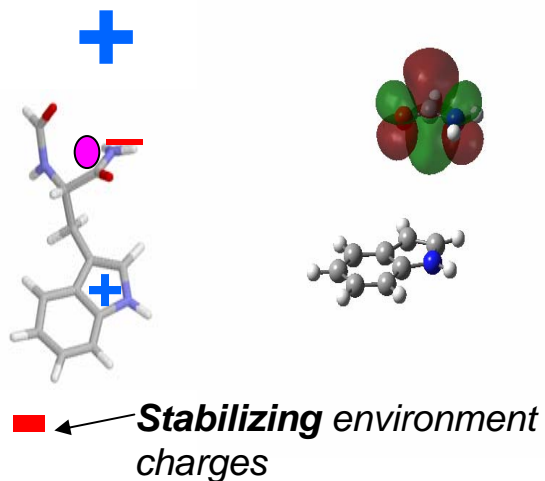
Explanation for large outlying points (in red box) from the comparison with experiment figure

These Trps each are buried and exhibit small relaxation, as in the case of RnaseT1 shown below. Our empirical scheme does not quite correctly capture the CT energy and its relaxation relative to the fluorescing state. If the relaxed energy were only 3 kcal above the 1L_a energy--instead of slightly below--the quantum yields would have been computed to be 0.3



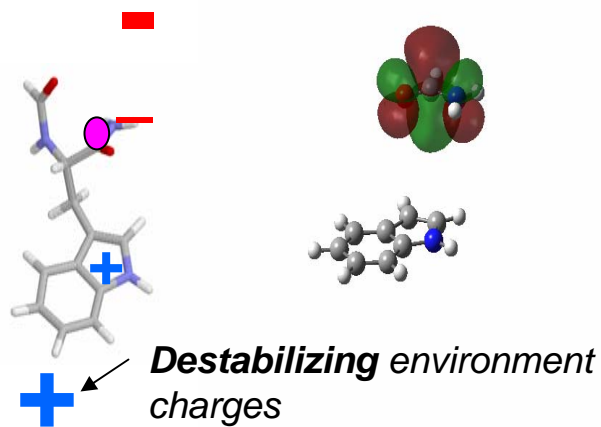
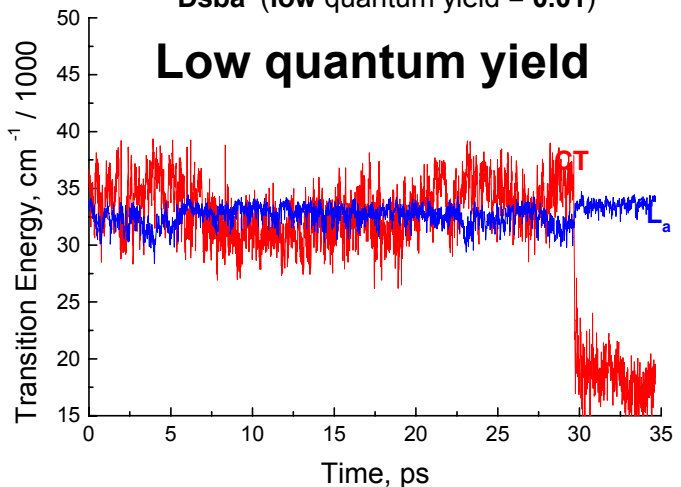
Summary

New Paradigm for Tryptophan Fluorescence Efficiency in Proteins:
Location is everything: No longer think of certain amino acids as electron acceptors. Instead, any charged amino acid can tune the energy gap between the usual fluorescing state and the charge transfer state on local amide. When the charge transfer (CT) state is stabilized, quenching occurs.



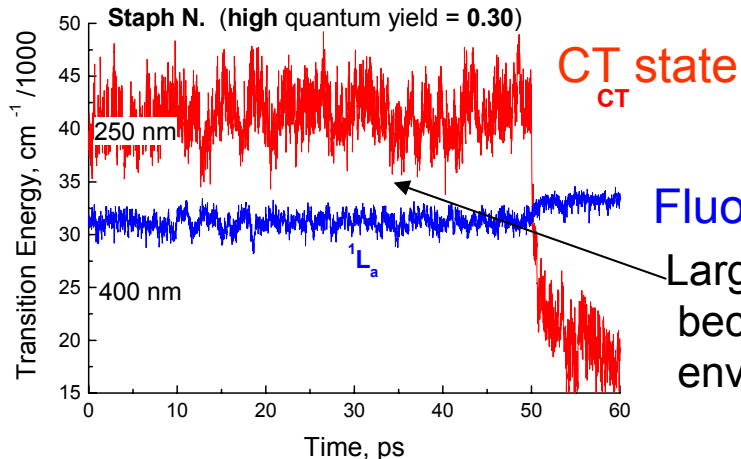
Dsba (low quantum yield = 0.01)

Low quantum yield



High quantum yield

La and CT Fluorescence Transition Energies for Staph N. (high quantum yield = 0.30)



At left are molecular dynamics trajectories

Summary (continued)

Semi-quantitative prediction of fluorescence quenching was achieved. This captures the basic phenomenon of extreme Trp fluorescence quantum yield (lifetime) variability in proteins.

The new underlying principle: location of charged groups can have a powerful influence on quenching by amide groups.

The reliable CASPT2 method strongly supports the semiempirical method used here.

Our goal is to evolve this work into a trusted method for ruling out or supporting postulated mechanisms and protein structural details.

Related and Future Work

Current work extends the method to other quenchers of Trp fluorescence, e.g., histidine cation, disulfide, cysteine, and methionine.

Work is in progress that will remove most of the empirical aspects of the predictive method presented here.