Photosynthesis Hot Paper

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Unraveling the Intrinsic Color of Chlorophyll**

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Abstract: *The exact color of light absorbed by chlorophyll (Chl) pigments, the light-harvesters in photosynthesis, is tuned by the protein microenvironment, but without knowledge of the intrinsic color of Chl it remains unclear how large this effect is. Experimental first absorption energies of Chl a and b isolated in vacuo and tagged with quaternary ammonium cations are reported. The energies are largely insensitive to details of the tag structure, a finding supported by first-principles calculations using time-dependent density functional theory. Absorption is significantly blue-shifted compared to that of Chl-containing proteins (by 30–70 nm). A single red-shifting perturbation, such as axial ligation or the protein medium, is insufficient to account even for the smallest shift; the largest requires pigment–pigment interactions.*

A number of variations on the basic chlorophyll (Chl) framework are employed by photosynthetic organisms. They all, however, contain a porphyrin with a magnesium ion (Mg^{2+}) in the center; only the peripheral substituents differ and whether one of the rings is reduced or not. Herein our focus is on Chl a and b (Figure 1), the most common chlorophylls found in plants. From their absorption spectra, it is evident that the molecules strongly absorb light in the red and blue regions of the visible spectrum (the Q and Soret bands, respectively), which in the absence of strongly colored secondary pigments is responsible for the green color of plant leaves. There are nevertheless significant differences between the spectra of Chl a and b, despite the fact that the two molecules only differ by one porphyrin ring substituent. The absorption spectra in ether solution display a difference of 232 nm between the Q band and Soret band maxima for Chl a whilst this is reduced to 189 nm for Chl b by a blue-shift of the Q band and red-shift of the Soret band.[1] Chl a and b in combination therefore provide broader coverage of the red and, more importantly, blue ends of the visible spectrum. Interaction with the surrounding microenvironment fine-tunes Chl to cover as much of the visible spectrum as possible.[2,3]

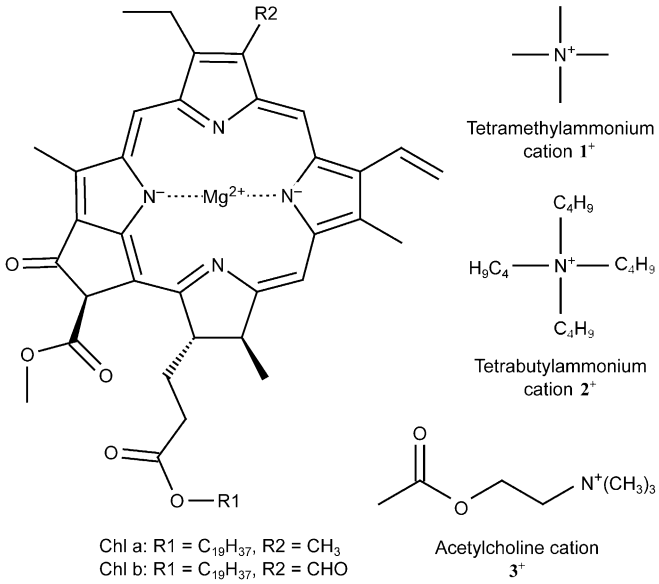

Figure 1. Structures of Chl a and b and charge tags ($\mathbf{1}^+$, $\mathbf{2}^+$, and $\mathbf{3}^+$).

A great amount of theoretical work has been directed towards understanding electronic absorption in various chlorophyll species.[4] Spectra of Chls dissolved in different solvents are abundant, but to make a rigorous comparison between experiment and theory, a more complete treatment of solvent effects in electronic structure calculations is needed.[5–7] Despite many developments in this field, excited-state calculations where some attempt is made to include such effects still inevitably possess a greater degree of uncertainty.[8–10]

It should also be borne in mind that the natural photosynthetic environment of a Chl molecule is usually a protein pocket, which is not necessarily well-modeled by bulk solvent. Thus absorption spectra of bare Chl molecules free of perturbing solvent interactions are a highly desirable goal.

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Multi-chromophore involvement in photosynthetic complexes is often dealt with using excitonic models; here excitons are linear combinations of local excited state wavefunctions (located on individual Chls), which implies that these states can exist in a spatially delocalized state on two or more Chls.[11–15] Knowledge of local excitation energies of individual pigments is therefore required for understanding exitonic coupling between Chls to address the concept of quantum coherence in photosynthesis.[12,3,16–19]

To assess the extent of modulation of Chl color within a protein microenvironment, it is necessary to know the free pigment color in the absence of perturbations from its surroundings. Indeed it can be difficult to conclude whether nearby amino acid residues are responsible for the color of one Chl, or whether it is instead due to electronic coupling with nearby Chls. Each photosystem contains up to several hundred Chls and to understand the complexity and local interactions is a formidable challenge. A good starting point is to unravel the photophysics of just one Chl molecule.

Neutral Chl molecules undergo rapid decomposition if evaporated for gas-phase spectroscopy. It is possible to deprotonate the molecules and study them in the gas phase by electrospray ionization mass spectrometry, as was recently demonstrated by Tanabe and co-workers.[20] However, spectral shifts are hard to interpret, as the electronic structures of the neutral molecule and molecular anions are different. We have taken a new approach (see the Supporting Information for methods) and tagged the molecules with the molecular cations tetramethylammonium, $\mathbf{1}^+$, tetrabutylammonium, $\mathbf{2}^+$, and acetylcholine $\mathbf{3}^+$ (see Figure 1). These charge tags possess no mobile protons, and it is therefore possible to know with certainty that the charge is located on the tag (quaternary ammonium group). The difference in bulk between $\mathbf{1}^+$ and $\mathbf{2}^+$ allowed the evaluation of the effect of altering the distance between the chlorophyll and the positive charge, and the more chemically complicated structure of $\mathbf{3}^+$ allowed the possibility of more complex interactions, for example with the magnesium center. Dissolved mixtures of Chl and charge tags were electrosprayed to produce complexes in vacuo, and photo-absorption was monitored using photodissociation mass spectroscopy.[27,28] A conventional absorption experiment based on the Lambert–Beer law was not possible in vacuo because the ion density was too low; instead absorption was monitored indirectly as a function of the dissociation rate of photoexcited ions using the technique of action spectroscopy.

Photoexcitation led to separation of the chlorophyll and charge tag at all excitation wavelengths. A representative photoinduced dissociation (PID) mass spectrum for Chl a·$\mathbf{3}^+$ is shown in Figure 2a obtained after irradiation with 600 nm light; the only significant fragment ion is $\mathbf{3}^+$. Dissociation resulted from two-photon absorption, evidenced by a quadratic dependence of $\mathbf{3}^+$ yield on laser pulse energy (Figure 2b). Similar results were found for Chl b and were independent of the cation tag. Therefore, to record absorption spectra, we monitored the yield of the cation tag versus excitation wavelength and divided the signal by the number of photons in the pulse squared. Spectra for Chl a·$\mathbf{3}^+$ and Chl b·$\mathbf{3}^+$ are shown in Figure 3a. In the case of Chl a, the

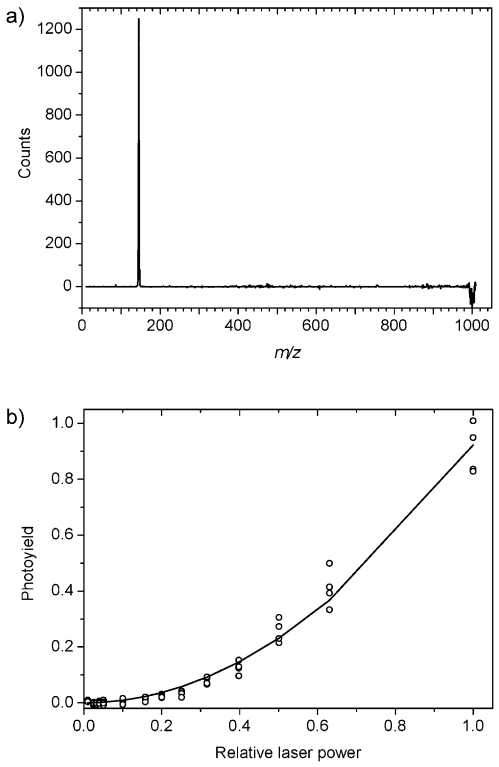

Figure 2. a) PID mass spectrum of Chl a·$\mathbf{3}^+$ ($\lambda = 600$ nm). The only relevant fragment ion observed is $\mathbf{3}^+$ (m/z 146). The scan was stopped before the m/z of the parent ion (m/z 1039) to avoid saturation of the detector. The signal at about m/z 1000 is from parent ions hitting the electrostatic analyzer at a too low (but well-known) electric field between the analyzer plates, and as a result the ions are reflected into the detector; the negative sign is expected for ion beam depletion due to photoexcitation. b) The photoyield of $\mathbf{3}^+$ versus relative laser power (maximum = 1) at 600 nm photoexcitation. The curve is an ax^2 fit to the data.

Soret band maximum is below 420 nm, while for Chl b the band is red-shifted with its maximum at about 445 nm. In contrast, the Q-band regions are blue-shifted for Chl b compared to Chl a, indicating that their difference is intrinsic and not due to the effects of microenvironment. The lowest energy-transition is at 637 nm and 627 nm for Chl a and b, respectively. Both spectra display vibronic transitions at higher energies and transitions owing to excitation along the other diagonal in the molecule.

Superimposed Q-band spectra of Chl a·$\mathbf{1}^+$, Chl a·$\mathbf{2}^+$, and Chl a·$\mathbf{3}^+$ are shown in Figure 3b. The spectra demonstrate negligible dependence on the nature of the charge tag. This suggests that the tag is located far enough from the π-electron density of the porphyrin ring system to have little effect on the absorption characteristics of the Chl molecule. To investigate this possibility further, first-principle calculations (see the Supporting Information) were performed on the three complexes, and it was found that the site energetically favored for location of the charge tag is adjacent to the oxygen atoms clustered near the phytyl tail of the Chl (Figure 4). Importantly, the tag was observed to move to this site when initially placed above the Chl magnesium atom where it also might have interacted in a favorable manner with the delocalized π-

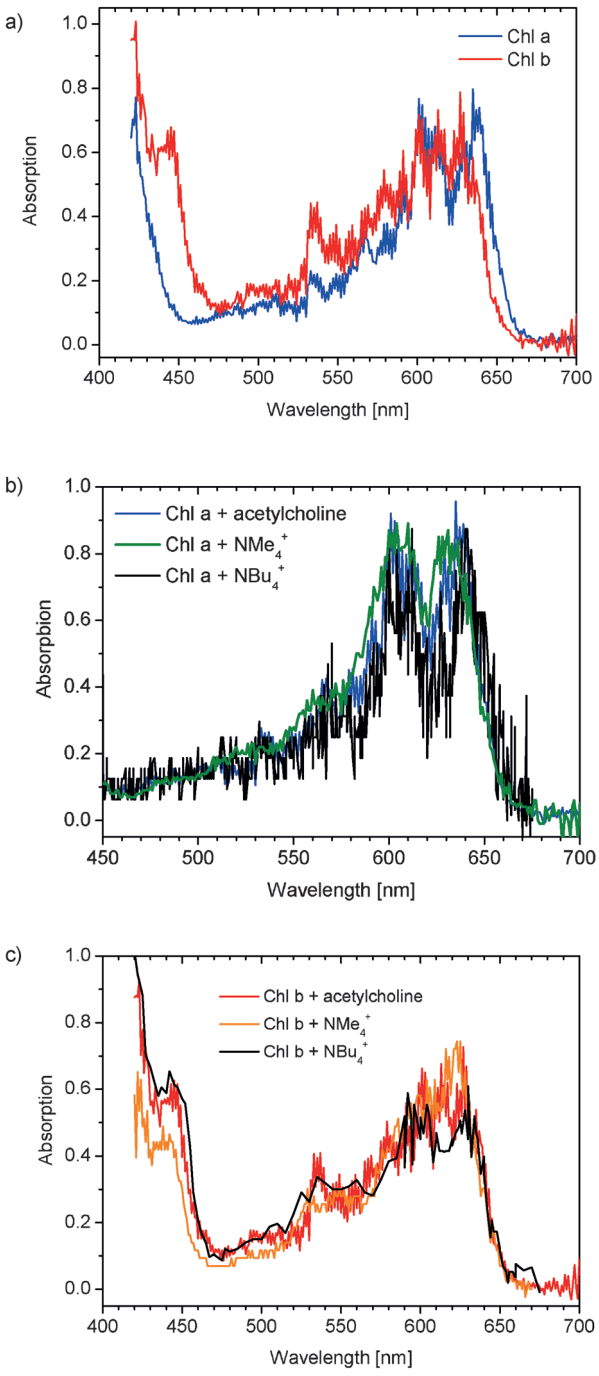

Figure 3. a) Absorption spectra of Chl a·$\mathbf{3}^+$ and Chl b·$\mathbf{3}^+$. b) Absorption spectra of Chl a·$\mathbf{1}^+$, Chl a·$\mathbf{2}^+$, and Chl a·$\mathbf{3}^+$. c) Absorption spectra of Chl b·$\mathbf{1}^+$, Chl b·$\mathbf{2}^+$, and Chl b·$\mathbf{3}^+$.

electron charge density. In the case of Chl a/b·$\mathbf{3}^+$, a structure was also identified where the $\mathbf{3}^+$ carbonyl oxygen was interacting with the magnesium center; this geometry was, however, higher in energy by 19 kJ mol^{-1} compared to the other complex in which no Mg–O interaction occurred. It was therefore assumed that the structure of Chl·$\mathbf{3}^+$ obtained in the experiments is the one with no binding to the magnesium center, in accordance with calculated isomer energies. Dissociation energies were calculated to be 131, 100, and 137 kJ mol^{-1} for Chl a·$\mathbf{1}^+$, Chl a·$\mathbf{2}^+$, and Chl a·$\mathbf{3}^+$, respectively.

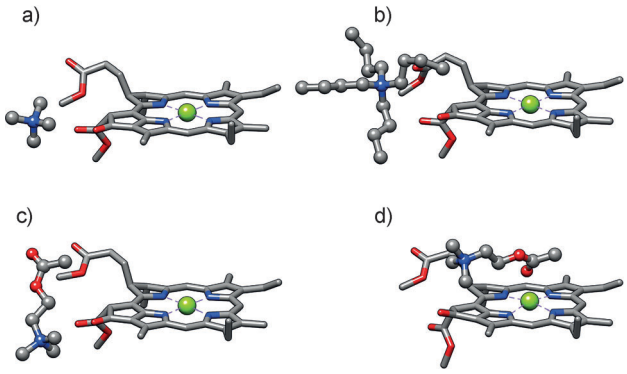

Figure 4. Optimized geometries of Chl a tagged with a) $\mathbf{1}^+$, b) $\mathbf{2}^+$, c) $\mathbf{3}^+$ but no Mg–O interaction, and d) $\mathbf{3}^+$ with Mg–O interaction. Chl a is shown in stick format, and charge tags in ball-and-stick format. Hydrogen atoms and the Chl phytyl tails have been omitted for clarity.

Complexes involving Chl b were very similar, with values of 123, 94, and 128 kJ mol^{-1}.

Calculation of the first excitation energy was performed for Chl molecules and charge tag complexes at optimized in vacuo ground-state geometries using time-dependent density functional theory (TDDFT).[21] The range-separated CAM-B3LYP functional[22,23] was chosen as this has previously been shown[24] to provide results similar to those obtained with the computationally demanding equation of motion coupled-cluster level of theory. TDDFT excitation energies are given in Table 1 together with the experimental band maxima. The results confirm that the tag has minimum influence on the excitation energy, with calculated values varying less than 0.03 eV for Chl a and 0.05 eV for Chl b (<10 nm). It should be mentioned that there are other cases where a charge tag plays minor role; for example, binding of metal cations or halide anions to arginine has little effect on its vibrational frequencies.[25,26]

The calculations systematically underestimated the experimental band maxima by 0.11 eV (ca. 30 nm) for Chl a and 0.15 eV (ca. 40 nm) for Chl b. Thus we corrected our theoretical values by subtracting 0.11 eV (0.15 eV), which allows us to obtain a values for the neutral chromophores. We find that the calculated excitation energy of Chl a is nearly identical to those of the complexes being 0.01 eV lower than that of Chl a·$\mathbf{2}^+$. The smallest deviation is for $\mathbf{2}^+$, where the distance from the cationic charge center to Chl a is largest. This rather small shift supports the experimental indications that the charge tag causes only minor perturbation of the Chl system.

Based on the combination of our experimental and calculated absorption data, we estimate that the Q-band absorption maximum of the isolated Chl a chromophore in vacuo would be 2.04 eV (theory)–0.11 eV (correction) = 1.93 eV (or 642 nm).

The spectra of Chl b complexes are of lesser quality than those of Chl a owing to lower ion beam currents. Despite this, it can be seen that the spectra are essentially independent of the nature of the charge tag, as with Chl a (Figure 3c). The lowest-energy band maximum changes from 630 nm for Chl b·$\mathbf{2}^+$ to 628 nm for Chl b·$\mathbf{3}^+$ to 623 nm for Chl b·$\mathbf{1}^+$,

Table 1: Summary of experimental band maxima and vertical transition energies (in eV) for the lowest-energy Q band.[a]

	1^+		2^+		3^+		No tag	
Exp. Chl a	1.97	(629)	1.94	(639)	1.95	(636)	1.93[d]	(642)
Theory Chl a	2.081	(596)	2.052	(604)	2.056[b]	(603)	2.041	(608)
					2.067[c]	(600)		
Exp. Chl b	1.99	(623)	1.97	(629)	1.98	(626)	1.98[d]	(626)
Theory Chl b	2.148	(577)	2.125	(584)	2.115[b]	(586)	2.129	(582)
					2.101[c]	(590)		

[a] Corresponding absorption wavelengths (in nm) are provided in parentheses. [b] No Mg–O interaction. [c] Mg–O interaction. [d] Estimation based on the deviation between theory and experiment.

which is the same trend that was seen for Chl a. Theory is once more in agreement with this (Table 1), but now overestimating the excitation energy by 0.15 eV (average value).

The estimated absorption energy for the bare Chl b is therefore (2.13–0.15) eV = 1.98 eV (626 nm).

In proteins the main $Q_y(0–0)$ absorption peak occurs between 660 nm and 680 nm, which is significantly red-shifted compared to our values for isolated Chl.[29] Axial ligand coordination has been shown to give a red-shift, the largest for negatively charged ligands (9–19 nm).[30] Combined axial ligation (< 20 nm) and non-specific protein interactions (perhaps 20 nm, based on solvent shifts) could explain this range. However, there are Chls in Photosystem I that absorb at even longer wavelengths (702–708 nm). Most likely these red absorptions are the result of exciton coupling between two or more Chls.

In the case of the Soret band, we can only conclude experimentally that the main peak is below 420 nm and therefore that solvation causes a red-shift. From our calculations the onset of Soret-band excitations occurred at about 365 nm for Chl a complexes, with the charge tags and at about 385 nm for the Chl b complexes. Corresponding excitations in the free chlorophylls occurred at 372 nm (Chl a) and 392 nm (Chl b).

In summary, the lowest excitation energies of Chl a and b in vacuo were found to be significantly blue-shifted compared to those measured in solution or photosynthetic proteins. This demonstrates unequivocally that environmental perturbations such as axial ligation and exciton coupling are red-shifting in nature. We also find that blue-shifting of the Q band of Chl b relative to Chl a is intrinsic to the molecules themselves and does not require exogenous interactions with the CHO moiety of Chl b for its explanation.

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