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Bonding Interactions in Anhydrous and Hydrated Chlorophyll a

Sir:

The currently accepted models[1-3] for anhydrous and hydrated Chl a aggregates are respectively given by (a) unsymmetrical Chl a-Chl a interactions involving the intermolecular C-9 keto C═O···Mg linkage[1,2] and (b) unsymmetrical Chl a-H_2O-Chl a interactions, C-10 ester C═O···HO(Mg)H···O═C keto C-9, involving the ring V cyclopentanone ester and keto carbonyls.[1,3]

A survey of the literature reveals that there are difficulties with both suggestions. Trifunac and Katz found[4] that lanthanide-induced-shift NMR observations demand an equivalence of the two Chl a molecules in the anhydrous dimer. They alluded to the difficulties of reconciling the unsymmetrical interaction (a) with the observed symmetry, and concluded that "a final detailed dimer structure still eludes" them.

The hydrated Chl a model (b) leads to the stoichiometry[1,3] (Chl a·H_2O)$_n$, and appears to be in conflict with the conclusions derived from the elemental analysis by Holt and Jacobs[5] and the x-ray study by Strouse[6] that the hydrated Chl a crystalline precipitate is the polymeric dihydrate (Chl a·$2H_2O$)$_n$.

In this communication, we propose that the probable molecular configuration for the anhydrous dimer is given by the symmetrical adduct shown in Figure 1, and offer experimental evidence that the long-wavelength (743 nm) absorbing Chl a-H_2O adduct is in fact most probably (Chl a·$2H_2O$)$_n$, in agreement with earlier analyses.[5,6]

Chlorophyll a was extracted from spinach and purified using standard procedures.[7] Absorption spectra of the purified Chl a in anhydrous ether (AR grade) solutions had absorption maxima at 4286 ± 1 and 6604 ± 1 Å with blue/red absorbancy ratios of 1.29 ± 0.01. These data have been consistently reproduced and they agree with established criteria for Chl a purity.[7] Solid Chl a, which under reduced pressure deposited from CCl_4 solution on the walls of a vessel, was heated under vacuum (~1 μ) at 75°C for time periods up to 24 hr. From room-temperature fluorescence intensity measurements, using the procedure after Livingston et al.,[8] it was found that hydrocarbon solutions prepared from samples heated for periods longer than 30 min at this temperature do not show any appreciable changes in the amounts of adventitious water. The two component solvents used in this study, methyl cyclohexane and *n*-pentane, were rigorously dried by distillation over $LiAlH_4$ in a dry nitrogen atmosphere. The solvent, a 1:1 mixture of rigorously dried methylcyclohexane and *n*-pentane, was transferred into sample tubes through vacuum-tight septa using syringes. A stock solution (suspension) of high purity water in methylcyclohexane was prepared by sonication. Aliquots of this solution were added along with the solvents to obtain solutions of known added water and Chl a concentrations. The solution employed in the present study contained 2 × 10^{-5} *M* Chl a.

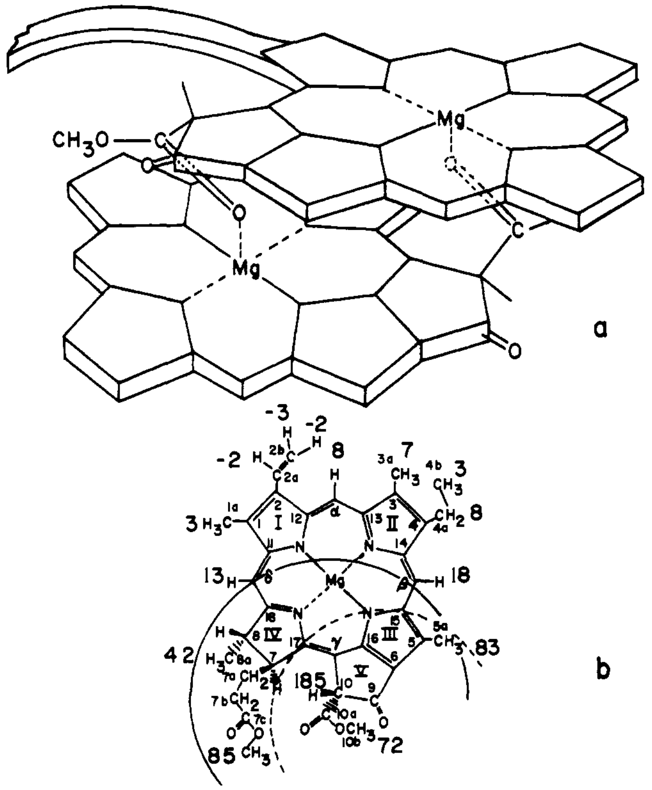

Figure 1. The C_2 symmetrical Chl a-Chl a adduct: (a) scaled model representation displaying the self-aggregating C-10 ester C═O···Mg linkages; (b) aggregation map of methyl chlorophyllide a from ref 1. The boldface numbers indicate the change in parts per million in the indicated resonance between dimer and monomer chlorophyll a. The solid semicircle centered on the ester carbonyl represents the circle of influence of the upfield ring current in the symmetrical structure shown in (a). The dashed semicircle which gives a poor fit of the upfield shifts at the protons of C-8a and the methoxy group at C-7c corresponds to the Katz model invoking the C-9 keto C═O···Mg linkage.

In agreement with Livingston et al.,[8] we find that the addition of water to hydrocarbon Chl a solutions activated the fluorescence intensity I_f of the chlorophyll. This intensity increases monotonically with increasing water concentration C_w. At $C_w = 3 \times 10^{-3}$ *M*, the value I_f for the 2 × 10^{-5} *M* Chl a solution is approximately half that of $I_{f,max}$ for the fully activated ($C_w = 1.5 \times 10^{-2}$ *M*) sample. The ratio $\eta = I_f/I_{f,max}$ (representing the fraction of Chl a in the form of the monohydrate[8]) is found to be 0.18 for the "dry" preparation. This result is in excellent agreement with the corresponding values obtained by Livingston et al.[8] who found that η ($\simeq$0.18) remains practically invariant in "dry" benzene solutions of Chl a ranging in concentration from 4.8 × 10^{-5} to 2.3 × 10^{-4} *M*. From water titration experiments using dilute ($C_0 \sim 10^{-6}$ *M*) Chl a solutions, in which apparently only the monomeric anhydrous and hydrated species, Chl a and Chl a·H_2O, are important, it was established[8] that the driest Chl a sample contained 0.5-1.0 molar equivalent of water.

The absorption spectra of six representative samples with varying amounts of added water are shown in Figure 2. The spectrum of the "dry" sample (Figure 2a) is qualitatively similar to that of a rigorously dried benzene solution of comparable Chl a concentration.[4] Two salient features of this spectrum are the main peak at ~663 nm (A663) and the shoulder at 678 nm (A678). The 678-nm shoulder is seen to diminish with increasing water concentration and apparently vanishes at $C_w \gtrsim 10^{-2}\ M$ (Figure 2e). The diminution of the 678-nm shoulder is accompanied by a corresponding increase in the 663-nm peak (Figures 2a-e). The interconversion of the 678-nm shoulder and the 663-nm peak thus manifests the dynamical equilibrium that exists between the anhydrous Chl a species and the hydrated species Chl a·H_2O, respectively.

Of particular interest is the observation of the A743 at high water concentrations. At $C_w = 2 \times 10^{-2}\ M$, a significant portion of the chlorophyll exists as the A743 in the freshly prepared solution (Figure 2f). At $C_w \gtrsim 5 \times 10^{-3}\ M$, the A743 appears as a major component upon aging of the sample (see Figure 2e,e′). In *all* cases, we observe that the appearance of the A743 is accompanied by the precipitation of Chl a, the latter presumably in the form of crystalline chlorophyll a dihydrate (chl a·$2H_2O)_n$.[5,6]

The appearance of A743 under excess-water conditions (Figure 2e′,f) is accompanied by a diminution of the A663, which we may ascribe to the equilibrium

$$n\text{Chl a} \cdot H_2O + nH_2O \overset{K_1}{\rightleftarrows} (\text{CHl a} \cdot 2H_2O)_n \qquad (1)$$

The monohydrate structure (Chl a·$H_2O)_n$ proposed[1,3] for A743 appears inconsistent with the results shown in Figure 2 in that the corresponding equilibrium

$$n\text{CChl a} \cdot H_2O \overset{K_2}{\rightleftarrows} (\text{Chl a} \cdot H_2O)_n \qquad (2)$$

does not depend on the water concentration.

The appearance of the A743 concomitant with the observation of the crystalline precipitate lends support to (1) because the crystalline precipitate of hydrated ethyl chlorophyllide a has been found[6] by x-ray diffraction analysis to be a polymeric aggregate of the ethyl chlorophyllide a dihydrate. The present finding is also consistent with the 1954 work of Holt,[6] who concluded from an elemental analysis that in crystalline hydrated chlorophyll, the chlorophyll exists as the dihydrate.

The experimental behavior of the hydration-dehydration of Chl a observed here and elsewhere[3,5,6,8-10] can be summarized as follows. The two waters of hydration in (Chl a·$2H_2O)_n$ are not equivalently held. Only the more loosely bound water of hydration can be readily set free under the conditions adopted in all the published drying procedures. This simple hypothesis accounts for the irreducible fluorescence first observed by Livingston et al.[8] and reproduced in the present work as well as the Ballschmiter and Katz observation[3] that the A743 aggregate yielded one water of hydration upon drying.[11]

The (Chl a·$H_2O)_n$ model[1,3] for A743 is based on the assumption that the driest available Chl a samples are completely anhydrous, which is contrary to earlier conclusions.[8,12,13] That the "anhydrous" Chl a solutions described in ref 1 contained water appears likely from an inspection of Figure 8 of that reference. In this figure, the 662 and 678 nm components of the absorption spectra of "anhydrous" hexane solutions of Chl a are attributed to the equilibrium

$$(662\text{ nm})n\text{Chl a}_2 \overset{K_3}{\rightleftarrows} (\text{Chl a}_2)_n(678\text{ nm}) \qquad (3)$$

If this were the case, the 678 nm/662 nm peak area ratio would depend sharply on the total concentration C_0 of Chl a according to the polynomial relationship

$$[(\text{Chl a}_2)_n] = K_3(\{C_0 - 2n[(\text{Chl a}_2)_n]\}/2)^n \qquad (4)$$

This dependence is not obtained in the sequence of five Chl a concentrations given in Figure 8 of ref 1, where we observe that the 678 nm/662 nm peak area ratio practically remains invariant over two orders of magnitude change (from 1.3×10^{-6} to $1.3 \times 10^{-4}\ M$) in C_0.

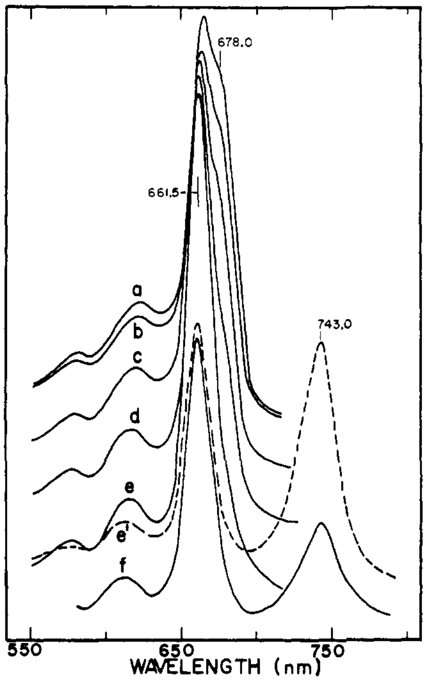

Figure 2. The effects of water titration on the room-temperature absorption spectrum of Chl a solution ($2.0 \times 10^{-5}\ M$) in 1:1 methylcyclohexane and *n*-pentane: (a) "dry"; (b) $4.0 \times 10^{-4}\ M$ added H_2O; (c) $2.0 \times 10^{-3}\ M$ added H_2O; (d) $7.0 \times 10^{-3}\ M$ added H_2O; (e) $1.0 \times 10^{-2}\ M$ added H_2O; (e′) $1.0 \times 10^{-2}\ M$ added H_2O; (f) $2.0 \times 10^{-2}\ M$ added H_2O. The solid curves are for freshly prepared samples. The dashed curve (e′) illustrates the spectral changes observed in the sample in (e) upon aging for 2 weeks in the dark at room temperature.

In reality, of course, one must incorporate the role of H_2O in order to explain the complex behavior. The assignment[1] of the 662-nm component to the anhydrous dimer Chl a_2 in the $1.3 \times 10^{-6}\ M$ solution appears to conflict with the work of Livingston et al.[8] who found that approximately 20% of the chlorophyll exists as Chl a·H_2O and ruled out dimer participation at $C_0 \sim 10^{-6}\ M$ in a detailed analysis of a large number of water titration experiments.

The structure (Figure 1a) for the anhydrous dimer Chl a_2 involves two reciprocal C-10 ester C=O···Mg linkages that are generically related to the symmetrical interactions proposed[14-16] for the primary molecular unit (Chl a·$H_2O)_2$ of P700, the photosystem I photoactive Chl a dimer that acts as the ultimate energy trap in photosynthesis.[17] These symmetrical interactions account for the observed[4] symmetry of Chl a_2 and appear to be consistent with the result[1] that Chl a oligomers are comprised of dimeric Chl a_2 units.

Infrared and NMR spectroscopic data offered[1] as evidence for models (a) and (b) have been interpreted respectively in terms of relative frequency and chemical shifts. These interpretations are often ambiguous. Sherman and Wang observed[9] that spectral changes in the 1600-1800-cm^{-1} region accompany the long-wavelength shift of the Chl a red absorption band upon hydration of the chlorophyll.[9,10] Ballschmiter and Katz[3] attributed the ir spectral changes to the unsymmetrical interaction (b). It appears that these changes are also consistent[18] with the bonding interactions deduced for the $(Chl\ a{\cdot}2H_2O)_n$ aggregate. The observed[19] downfield ^{13}C NMR effect observed for C-9 is thought to provide unequivocal evidence for the unsymmetrical interaction (a). However, reexamination[18] of the ^{13}C NMR data of Boxer et al.[20] reveals that the C-10 resonance has apparently undergone an even larger shift upon Chl a aggregation.[22]

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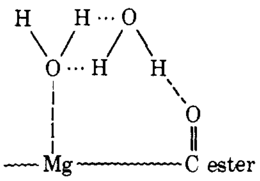
Ester and Keto Carbonyl Linkages in Chlorophyll a, Pyrochlorophyll a, and Protochlorophyll a

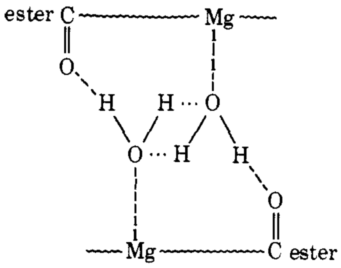
Sir:

In recent work, we concluded[1] that the existing experimental observations[2,3] relevant to the primary light reaction can be interpreted on the basis of the C_2 1:1 $(Chl\ a{\cdot}H_2O)_2$ adduct structure in which two chlorophyll molecules are held by two complementary ester C=O···HO(Mg)H linkages. This conclusion presents an alternative to that of the earlier Katz-Norris postulate that the reaction center "special pair"[2c] consists of two chlorophyll a molecules interlinked by a single water molecule through the unsymmetrical interaction ester C=O···HO(Mg)H···O=C keto. Independently and simultaneously, Strouse[4] found from single-crystal x ray work that the unit interactions in ethyl chlorophyllide $a{\cdot}2H_2O$ polymeric aggregate are given by



A

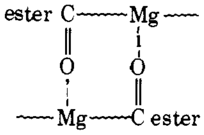
(See Figure 1 of ref 4). We observe that a symmetrical addition of a second Chl a molecule to A results directly in the self-aggregating interactions proposed[1] for the symmetrical $(Chl\ a{\cdot}H_2O)_2$ adduct.



B

In both A and B, the coordination interaction occurs on the same side of the chlorin plane as the C-10 ester group, thus taking advantage of the 0.4 Å displacement of the Mg atom in the same direction as the ester group.[4] The Katz structure invokes water coordination from the direction *opposite* to that of the Mg atom displacement.

In a reexamination of the current literature on the infrared[5] and NMR[6] spectra of Chl a, it has been pointed out[7] that self-aggregating ester C=O···Mg linkages may play an important role in Chl a dimerization. A probable structure for the Chl a dimer can be given by the bonding interactions:[7]



C

We note that in C the self-aggregating interactions again take advantage of the out-of-plane displacements of the Mg atoms. Structure C is a modification of the earlier conclusion that the Chl a dimer occurs through the asymmetrical keto C=O···Mg linkage. Both the present model C and the Ballschmiter–Katz model are distinctly different from yet another model by Houssier and Sauer[8] who proposed that Chl a dimerization involves both the C-9 keto oxygen and the propionic ester at C-7. In this communication, we attempt to evaluate the validity of the various dimer models.

In pyrochlorophyll a, no ester C=O···Mg interactions are allowed due to the absence of the ring V carbomethoxy group. The upfield ^1H NMR shift difference[9] (produced by intermolecular aggregation) of the proton at C-10 is 50 ppm. This value is remarkably lower than the corresponding shift difference 185 ppm observed in chlorophyll a,[2c,6a] and reflects the probable role of the carbomethoxy carbonyl group in the dimerization of Chl a molecules.

From a qualitative comparison of the optical properties of dimeric Chl a, and pyroChl a and protochlorophyll a (PChl a), Sauer and co-workers[8] arrived at the conclusions (i) that the ring V carbomethoxy group has no significant influence on the dimer structure and (ii) that the C-7 propionic ester and the C-9 keto function are both involved in dimerization. These conclusions were based on the fact that while the circular dichroism (CD) and absorption spectra of dimeric Chl a and pyroChl a are "similar", the corresponding spectrum of PChl a is dramatically different.[10]

A reexamination of Houssier and Sauer's data reveals that the spectroscopic differences and similarities between the dimers of Chl a, pyroChl a, and PChl a appear to be a matter of degree. The CD spectrum of PChl a is different from those of Chl a and pyroChl a, but the CD spectra of Chl a and pyroChl a dimers are less so. (Compare, for ex-