## ANNALES

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## Hydrogen Bonding and Disolvation of Chlorophyll A in Mixed Basic and Protic Solvents

Wiązania wodorowe i bisolwatacja chlorofilu A w mieszanych rozpuszczalnikach zasadowych i protonowych

#### INTRODUCTION

The factors that influence the absorption spectra of chlorophyll (Chl) have been considered in many studies in relation to various spectral forms of Chl in vivo. Exciton theories that include both transition-dipole interactions in neighbouring Chl molecules and ad hoc assumed environmental shifts have been developed in order to fit the theoretical predictions to experimentally determined spectra, even for pigment-proteins of exactly known molecular structure [7, 9, 12, 16]. Among the presumed non-excitonic effects, which may be of very different nature [4-6, 13], two are closely related to intrinsic properties of Chl molecules. These are the hydrogen bonding at the keto C=O group conjugated with the electronic pi-system, and coordination interactions at the central magnesium atom in Chl molecule, (explained in Figure 1). Recently, interactions of both types were investigated in detail under assumption that they are independent of nonspecific (solvatochromic) effects which always occur in polarizable media [10]. Specific electron--donor interactions at the Mg atom in Chl in basic solvents were found to be effective in shifting the  $Q_{\nu}(0-0)$  absorption band only when Mg is coordinated externally with two ligands containing nitrogen atoms, while 6-coordination with oxygen ligands was found ineffective. On the other hand, hydrogen bonding in protic solvents has been found to produce bathochromic shifts of  $Q_{u}(0-0)$ , proportional to changes in vibrational frequency of the keto C=O group, as determined by resonance Raman (RR)

spectroscopy [10]. However, in two protic solvents methanol and aniline, partial 6-coordination was noted in addition to hydrogen bonding, and extra red shifts in absorption spectra were found that are apparently due to interference of both these interactions. The present study concentrates on absorption and RR spectra of Chl in mixed basic and protic solvents. It was undertaken in order to examine the effect of hydrogen bond formation at the keto C=O group on the position of  $Q_y(0-0)$  band in absorption spectra of Chl with 6-coordinated Mg atom.



Fig. 1. Molecular structure of chlorophyll a. The dashed line indicates the system of conjugated double bonds. The groups involved into spectroscopically relevant molecular interactions are (i) the magnesium atom in its center, plane coordinated by the four nitrogen atoms of pyrrole rings I-IV and available for the fifth and sixth external ligands perpendicular to the molecular plane, and (ii) the keto oxygen atom at carbon C<sub>9</sub>, capable to form hydrogen bond with proton donors

Ryc. 1. Struktura molekularna chlorofilu a. Linia przerywana wskazuje układ wiązań sprzężonych. Grupami zaangażowanymi w spektroskopowo istotne oddziaływania molekularne są: (1) atom magnezu w centrum molekuły, płasko koordynowane przez cztery atomu azotu pierścieni pirolowych I-IV i dostępne dla piątego i szóstego zewnętrznych ligandów prostopadłych do płaszczyzny molekuły, oraz (2) atom tlenu w grupie ketonowej przy C<sub>9</sub>, tworzący wiązania wodorowe z donorami protonów

### MATERIALS AND METHODS

Chlorophyll a was extracted from nettle leaves and was purified by high-performance liquid chromatography following the published procedures [17] with small modifications [10]. Visible absorption spectra were recorded on Specord UV VIS and M40 instruments. Two wavelengths, 441.6 nm and 457.9 nm, from He-Ne and  $Ar^+$  lasers were used to obtain the RR spectra from spinning samples. The scattered light was analysed by a Raman spectrometer based on a double-grating monochromator and a photon counting system. Chl concentrations used were 0.5 mM for absorption and 2 mM for Raman spectroscopy. All experiments were performed at temperature 22°C.

### RESULTS

#### **RESONANCE RAMAN SPECTRA**

Phenol was used as hydrogen bond donor because of its strong effect on absorption spectra through hydrogen bonding and its inability to form 6-coordinated Chl [10]. With each basic solvent, several RR spectra were recorded for samples differing in the molar ratio phenol/base. Due to phenolbase hydrogen bonding, low concentrations of phenol were ineffective in producing hydrogen bond Chl. High proportion of phenol, however, resulted in 5-coordinated Chl due to deficiency of the base. Optimum molar ratios were 1:1 for mixtures of phenol with tetrahydrofuran and pyridine and 3:10 for dioxane/phenol. Additionally, acetone was used as reference 5coordinating basic solvent in 3:10 molar ratio to phenol.

The Soret absorption band in the violet shifts to longer wavelengths similarly to the  $Q_y(0-0)$  band [15]. Thus, excitation of RR spectra at two wavelengths in the region of Soret band provides information about the spectral inhomogeneity of Chl in mixed solvents, since with the longer--wavelength light (457.9 nm) the resonance enhancement of more red-shifted Chl species is obtained.

The resonance Raman spectra recorded in mixed solvents are given in Figures 2-5. In dioxane and in dioxane-phenol mixture, the three bands at 1522, 1546 and 1596 cm<sup>-1</sup> indicate 6-coordination in accordance with the established criteria [8,10], since these frequencies are by  $\cong$  9,  $\cong$  9 and  $\cong$  15 cm<sup>-1</sup> lower than for 5-coordinated Chl. The free keto C=O band at 1696 cm<sup>-1</sup> in dioxane shifts to 1670 cm<sup>-1</sup> and 1666 cm<sup>-1</sup> in dioxane-phenol due to hydrogen bonding with phenol. In pure phenol, the C=O band appears at slightly lower frequency 1662 cm<sup>-1</sup> [10]. On the other hand, the frequency of free keto C=O must lie above 1686 cm<sup>-1</sup> [10]. Thus, two slightly different values of  $\nu_{C=O}$  under 441.6 nm and 457.9 nm excitation (1670 and 1666 cm<sup>-1</sup>, respectively) reflect some differences in local polarizability around hydrogen bond in the mixed solvent rather than incomplete hydrogen

bonding. Essentially the same is indicated by RR spectra in tetrahydrofuran--phenol in Figure 3. However, the high-frequency shoulder in the keto C=O band at 1668 cm<sup>-1</sup> in Figure 3 reveals that a small fraction of keto groups remains free in this case.



Fig. 2. Resonance Raman spectra of chlorophyll *a* in dioxane and in dioxane/phenol (3:10). Excitation wavelengths in nanometers are indicated at each spectrum Ryc. 2. Rezonansowe widma ramanowskie chlorofilu *a* w dioksanie i w mieszaninie dioksan/fenol (3:10). Długości fali wbudzającej są zaznaczone przy każdym widmie



Fig. 3. Resonance Raman spectra of chlorophyll *a* in tetrahy-drofuran and tetrahydrofuran/phenol (1:1)

Ryc. 3. Rezonansowe widma ramanowskie chlorofilu a w czterohydrofuranie i w mieszaninie czterohydrofuran/fenol (1:1)

The differences between the frequencies of hydrogen bond C=O in dioxane-phenol (1666 cm<sup>-1</sup>), tetrahydrofuran-phenol (1664 cm<sup>-1</sup>) and that in phenol (1662 cm<sup>-1</sup>) are not large and can be fully related to lower polarizability of these mixtures which always leads to higher C=O frequencies than those in pure phenol [10]. This conclusion is confirmed by the *RR* spectrum of Chl in acetone-phenol in Figure 5 (acetone is a 5-coordinating solvent). The identity of the keto C=O frequency in this mixture with that in pure phenol is due to the high dielectric constant of acetone which compensates for its low refractive index.



Fig. 4. Resonance Raman spectra of chlorophyll *a* in pyridine and in pyridine/phenol (1:1). Excitation wavelengths in nanometers are indicated at each spectrum

Ryc. 4. Rezonansowe widma ramanowskie chlorofilu *a* w pirydynie i w mieszaninie pirydyna/fenol (1:1). Długości fali wzbudzającej są zaznaczone przy każdym widmie





Ryc. 5. Rezonansowe widma ramanowskie chlorofilu a w acetonie i w mieszaninie aceton/fenol (3:10)

Unlike to the above, the RR spectrum in pyridine-phenol (Fig. 4) under 441.6 nm excitation exhibits a remarkable splitting of the keto C=O band into two components of which the one at 1688 cm<sup>-1</sup> is exactly the same as in pure pyridine [10], and the other, at 1660 cm<sup>-1</sup>, almost closely corresponds to hydrogen bond C=O in pure phenol (1662 cm<sup>-1</sup> [10]). This picture indicates that hydrogen bonding in pyridine-phenol is highly incomplete, even at the optimum ratio of both components. Generally, this is consistent with the much higher value of the equilibrium constant for the formation of hydrogen bond complexes of phenol with pyridine than those of phenol with oxygen bases [11]. For phenol-pyridine, this equilibrium constant (in carbon tetrachloride) is 52 l/M [11] and it may be calculated that only about 5 percent of both components remain free at 1:1 molar ratio of reagents.

The 6-coordination of Mg atom in dioxane-phenol and in tetrahydro-

furan-phenol was complete (see above). However, again it is not the case in pyridine-phenol, as indicated by slightly higher C = C frequencies (cf. Figs. 2-4) and by the disappearance of the 1595/1610 cm<sup>-1</sup> band due to its splitting into 5- and 6-coordinate components and by the overlap with the weak shoulder at  $\approx 1630$  cm<sup>-1</sup>.



Fig. 6. Postulated scheme of molecular interactions through hydrogen bonding (dotted lines) in methanol which result in increased basicity of oxygen atoms coordinating the magnesium atom in the chlorophyll molecule

Ryc. 6. Schemat postulowanych oddziaływań molekularnych poprzez wiązania wodorowe (linie kropkowane) w metanolu, które prowadzą do wzrostu kwasowości atomów tlenu koordynujących atom magnezu w molekule chlorofilu

#### ABSORPTION SPECTRA

It has been shown in a previous study [10] that the absorption wavelength at the maximum of the  $Q_y(0-0)$  band follows the relation:

$$\lambda = \lambda_0 + K \cdot X(n,\epsilon) + \Delta \lambda_s, \tag{1}$$

where  $\lambda_0 = 645.8$  nm is the extrapolated absorption wavelength in vacuum, K = 38.2 nm is a constant,  $\Delta \lambda_s$  stands for the spectral shift due to specific interactions ( $\Delta \lambda_c$  for coordination,  $\Delta \lambda_{hb}$  for hydrogen bonding).  $X(n, \epsilon)$  is a function of solvent's refractive index and dielectric permittivity emerging from the theory of solvatochromism [1]:

$$X(n,\varepsilon) = \frac{n^2 - 1}{2n^2 + 1} + 0.73 \frac{2n^2 + 1}{n^2 + 2} \left[ \frac{n^2 - 1}{n^2 + 2} + 0.058 \left( \frac{\varepsilon - 1}{\varepsilon + 2} - \frac{n^2 - 1}{n^2 + 2} \right) \right].$$
(2)

Thus, assuming the same coordination state of Chl in base-phenol and in pure base solution, and assuming that the spectral shifts related to hydrogen bonding and coordination are additive, we can write:

$$\lambda_b = \lambda_0 + K \cdot X(n,\varepsilon) + \Delta \lambda_c \tag{3}$$

for absorption wavelength in the pure base, and:

$$\lambda_m = \lambda_0 + K \cdot X(n_m, \varepsilon_m) + \Delta \lambda_c + \Delta \lambda_{hb} \tag{4}$$

for absorption wavelength in base-phenol mixture. It follows from Eqns. (3) and (4) that:

$$\Delta \lambda_{hb} = (\lambda_m - \lambda_b) - K \cdot [X(n_m, \varepsilon_m) - X(n, \varepsilon)].$$
(5)

The values of refractive index  $n_m$  and dielectric permittivity  $\varepsilon_m$  of mixed solvents were calculated from the Clausius-Mossotti equation [3] and, after substituting them into Eqn. 5, the values of  $\Delta \lambda_{hb}$  were calculated. They are included together with other relevant parameters in Table 1.

### DISCUSSION

As can be seen in Table 1, the values of  $\Delta \lambda_{hb}$  for chlorophyll hydrogen bonded with phenol and simultaneously 6-coordinated with dioxane or tetrahydrofuran (both providing two oxygen ligands at Mg) are similar to  $\Delta \lambda_{hb}$  in pure phenol. In mixed solvent acetone-phenol (5-coordinated Mg),  $\Delta \lambda_{hb}$  is also similar to that in phenol and this finding confirms the validity of the method used for estimating  $\Delta \lambda_{hb}$  values based on Eqn. 5. Thus, the spectral effects of hydrogen bonding do not depend on possible changes in Chl's electronic structure introduced by Mg coordination (out-of-plane position of Mg when 5-coordinated and in-plane position of Mg when 6-coordinated). For this reason, extra red shifts in hydrogen bonded and 6-coordinated Chl in methanol and aniline [10] should not be ascribed to the interference of both these effects but rather to the specific chemical nature of the externel ligands coordinating the magnesium atom. Based on this, the question concerning the extra red shifts in methanol and in aniline can be solved by considering molecular interactions in these solvents. It is known that hydrogen bonding leads to an increase of negative charge on nucleophilic atoms in hydrogen bond donor groups [2, 14]. Thus, the basicity of oxygen atoms in coordinating methanol molecules is increased due to their involvement into hydrogen bonds with other methanol molecules as illustrated in Figure 6, and this results in an additional red shift of the  $Q_y$  absorption band. The same mechanism is postulated here also for aniline in which the largest value of  $\Delta \lambda_s$  has been found [10]. Since the basicity of the coordinating ligands is relevant for band shifts in absorption spectra only in 6-coordinated state of the Mg atom [10], it is clear that the same phenomenon in other

protic solvents cannot result in extra red shift because of 5-coordination in these solvents.

Table 1. Coordination numbers of magnesium atom in Chl, keto C=O frequencies, refractive indices and dielectric permittivities of solvents, absorption wavelengths and  $\Delta \lambda_{hb}$  values for chlorophyll  $\alpha$  in pure and mixed solvents. Parentheses indicate partial hydrogen bonding and 6-coordination. The data for phenol were obtained at 50°C, i.e. in melted phenol

Tab. 1. Liczby koordynacyjne atomu magnezu w Chl, częstości grup keto C=O, współczynniki załamania i stałe dielektryczne rozpuszczalników, długości fali pasma absorpcji i wartości  $\Delta \lambda_{hb}$  dla chlorofilu *a* w rozpuszczalnikach czystych i mieszanych. Nawiasy wokół liczb oznaczają częściową 5- i 6-koordynację lub częściowy udział wiązań wodorowych. Dane dla fenolu otrzymano w 50°C (tj. w stanie ciekłym)

Solvent	Coord. number	$\nu_{\rm C=0}  [\rm cm^{-1}]$	n	ε	$\lambda$ [nm]	$\Delta \lambda_{hb} \ [nm]$
dioxane	6	1697	1.4221	2.2	662.0	—
dx/ph (3:10)	6	1666	1.5130	6.6	670.2	4.5
tetrahydrof.	6	1694	1.4040	7.4	665.0	—
tf/ph (1:1)	6	1664	1.4714	8.6	672.7	5.4
pyridine	6	1688	1.5092	12.3	671.0	-
py/ph (1:1)	(6)	(1660)	1.5243	11.1	673.2	1.8
acetone	5	1691	1.3591	20.7	661.9	_
ac/ph (3:10)	5	1662	1.4973	11.1	671.5	5.1
phenol	5	1662	1.5403	10.3	673.1	5.5

Basic solvents used in this study possess nucleophilic atoms whose basicity cannot be modified by e.g. hydrogen bonding and thus they allow to recognize the specific effects of protic solvents in more detail.

Incomplete 6-coordination and hydrogen bonding in pyridine-phenol hinders the quantitative analysis of their spectral effects. Nevertheless, an indication of additional spectral shifts related to hydrogen bonding can be inferred also in this case from  $\approx 2$  nm value of  $\Delta \lambda_{hb}$ .

In conclusion, the analysis of RR and absorption spectra of Chl in neat and mixed solvents confirms that the spectral shift related to hydrogen bonding is independent of the coordination number of the Mg atom in the Chl molecule. It also proves that the increased basicity of the Mg-coordinating atom in the solvent molecule can significantly shift the  $Q_y(0-0)$  absorption band of Chl in 6-coordinated state.

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#### STRESZCZENIE

Niniejszy artykuł przedstawia analizę widm absorpcyjnych i rezonansowych widm ramanowskich chlorofilu *a* w mieszanych rozpuszczalnikach zasadowych i protonodonorowych, wykonaną w celu ustawienia związku przesunięć spektralnych z typem oddziaływania molekularnego. Wiązania wodorowe i oddziaływanie koordynacyjne wpływają na położenie pasm absorpcyjnych niezależnie od siebie. Stwierdzono, że wyjątkowo duże przesunięcia pasm ku czerwieni w niektórych rozpuszczalnikach (metanol, anilina) wywolane zostały koordynacją molekuł chlorofilu przez silnie zasadowe atomy nukleofilowe zaangażowane w poboczne wiązania wodorowe z rozpuszczalnikiem.