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Interplay between Acetate Ions, Peripheral Groups, and Reactivity of the Core Nitrogens in Transmetalation of Tetrapyrroles

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Abstract: The mechanism of acetate-assisted transmetalation of tetrapyrroles was investigated in a model system consisting of chlorophyll a and copper(II) acetate in organic solvents by using a spectroscopic and kinetic approach. Surprisingly, acetate ions bind to the central Mg in chlorophyll much more strongly than do acetonitrile, methanol and even pyridine, one of the best ligands in chlorophyllic systems. This exceptionally strong non-symmetrical axial ligation of the central Mg by acetate causes its out-of-plane displacement and deformation of the tetrapyrrole ring, thus facilitating the interaction with an incoming Cu^{II} complex. This mechanism is controlled by a keto–enol tautomerism of the chlorophyll isocyclic ring. Additionally, depending on solvent, acetate activates the incoming metal ions. These new insights allow to suggest a mechanism for the acetate method of metal exchange in tetrapyrrolic macrocycles, which resembles biological insertion of metal ions into porphyrins. It also provides a guideline for the design of more efficient methods for the metalation of porphyrins and related macrocycles.

Keywords: acetate effect · chlorophylls · keto–enol tautomerism · porphyrinoids · transmetalation

Introduction

Metalloporphyrins are key cofactors in many catalytic systems of biological and synthetic importance and since long the methods of metal insertion and exchange in tetrapyrroles are of great interest. There are two general paths towards metal exchange in tetrapyrrolic complexes. Under acidic conditions, the transmetalation of metalloporphyrins and chlorophylls (Chls) proceeds via dissociation of the central metal followed by a reaction of the free base with the incoming metal cation. In the absence of acid, the exchange of metal ion proceeds via an interchange mechanism. Numerous studies on metalloporphyrins have shown that there are several factors which determine the kinetics of transmetalation, such as the coordination properties of the metal ion, its ligands and the nature of the solvent.

The structural factors which determine the properties of the porphyrin/Chl macrocycle are also important.[1–6] In particular, structural differences between porphyrins and Chls (Figure 1) seem very relevant, but they have not yet been compared in the context of their abilities to control the exchange of the central metal ion. The transmetalation of Chls is a reaction in which the central Mg ion is displaced by another divalent metal ion, as shown in Equation (1).

$$\text{Mg-Chla} + M^{II} \rightarrow \text{Mg} + \text{M-Chla} \quad (1)$$

The substitution in some cases, for example, with Zn^{II} and Cu^{II}, occurs spontaneously but usually is irreversible, as reported by Schunck and Marchlewski already at the end of the 19th century.[7] These spontaneous reactions pose a serious threat in natural systems as they may cause the irreversible loss of photosynthetic activity, for example, in plants

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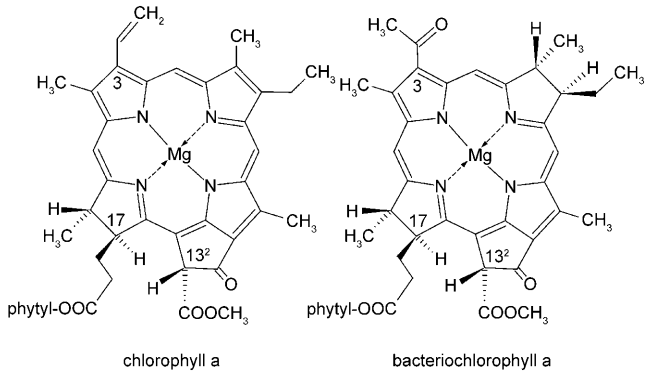

Figure 1. Chemical structures of chlorophyll a and bacteriochlorophyll a, major photosynthetic pigments of higher plants and purple photosynthetic bacteria, respectively.

growing on soils polluted by heavy metals.[8] On the other hand, metal displacement is of great interest when considering the use of metallosubstituted Chls and bacteriochlorophylls (BChls) in model studies[9–13] and their application as photosensitizers in photodynamic therapy (PDT).[14–23] This has lead to the development of methods to achieve an insertion of divalent metal ions that cannot spontaneously replace the central Mg^{2+}.[24] Among these the so-called "acetate method" is commonly used in the synthesis of metallosubstituted porphyrins and Chls.[1,25,26] Recently, modifications of this general method were employed to synthesize for the first time the Zn- and Cu-substituted BChla[5] and Pt-substituted Chla.[27] Although the method is already for decades in use, its mechanism remains ill-defined.[1,3] The present study is aimed to examine the underlying mechanism of the metal exchange reactions of chlorophylls and its activation by acetate.

In this report we show that the effect of acetate ions on the transmetalation of Chla depends strongly on the coordination properties of the solvent. Moreover, the activating effect of acetate (Ac) seems intrinsically linked to the occurrence of the keto–enol tautomerism at the isocyclic ring of Chl. Spectrophotometric titrations of BChla with tetrabutylammonium acetate (TBAAc), 2,2′,2″,2‴-(ethylenedinitrilo)-tetraethanol (THEED) and pyridine (py) were performed in order to determine the forms of pigment coordination in the presence of these chelators. Because Ac ions, solvent molecules and Chl can all act as potential ligands for Cu^{II} and Mg, we considered all the possible and essential equilibria that can occur in this system. In addition, the kinetics of the transmetalation of Chl in the presence of TBAAc and THEED was investigated using conventional kinetic techniques.

Results and Discussion

Interaction of acetate ions with chlorophylls: In order to determine the possible non-symmetrical axial interaction of Chla with chelating ligands, titrations of BChla in weakly ligating MeCN with py, TBAAc and THEED were performed. The use of BChla was motivated by the better distinction of the coordination-sensitive Q_X transition in this pigment.[28,29] According to the available data,[29,30] BChla and Chla are mainly five-coordinate in MeCN and six-coordinate in MeOH. Whatever the solvent, it can be replaced by py, which is known to force Chls to form symmetrical six-coordinate complexes.[30,31] In this context, BChla was incubated in MeCN containing 0.15 M py and then small doses of 0.5 M TBAAc or THEED solutions were added. Every addition increased the concentration of TBAAc or THEED by 0.02 M. Figure 2A shows the relevant region of the absorption spectrum of BChla pre-incubated with py, recorded during titration with TBAAc and THEED, whereas Figure 2B shows the Q_X band of BChla pre-incubated with TBAAc or THEED and then titrated with py.

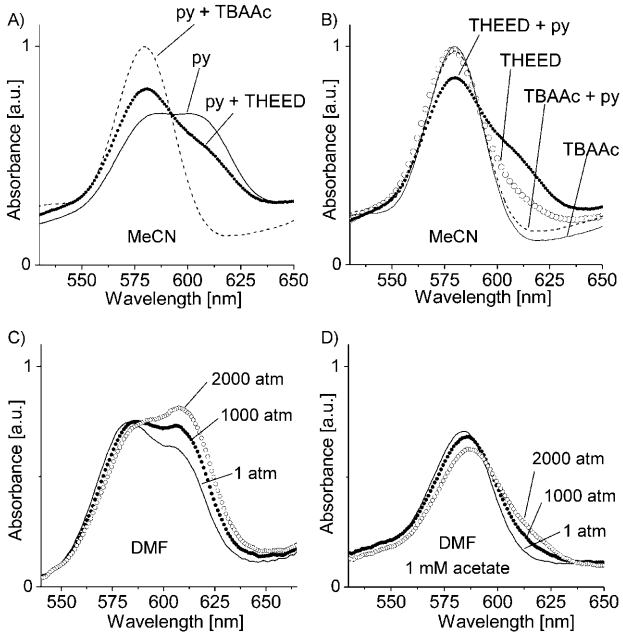

Figure 2. Effects of various chelators and high pressure on the shape of the coordination state-sensitive Q_X band of bacteriochlorophyll a (2.5×10^{-6} M) in acetonitrile (MeCN) and in dimethylformamide (DMF), respectively. A) To the solution of the pigment pre-incubated with 0.15 mM pyridine (solid line), 0.02 M 2,2′,2″,2‴-(ethylenedinitrilo)tetraethanol (THEED, •••••) or 0.1 M tetrabutylammonium acetate (TBAAc, -----) was added. B) The pigment in MeCN was first treated with THEED (○) or with TBAAc (——) and then pyridine was added (dotted and dashed line, respectively). The same concentrations were applied as in A). C) and D) High pressure-induced changes in the shape of the Q_X band of bacteriochlorophyll a in neat DMF and in DMF containing 1 mM sodium acetate, respectively.

In the presence of 0.15 M py in MeCN an equilibrium between five- and six-coordinate forms of BChla exists, as inferred from the appearance of the two-component Q_X band (solid line in Figure 2A). Upon the addition of TBAAc, the component near 610 nm disappears while the one at 580 nm increases significantly. The addition of THEED under the same conditions causes only a partial shift towards the

580 nm band (Figure 2A). No significant spectral changes were observed when py was used as the titrant for a solution of BChla pre-incubated with TBAAc (Figure 2B), whereas the final spectrum of BChla in MeCN/THEED titrated with py was comparable with that recorded during the titration of BChla/py with THEED. In strongly coordinating MeOH, the Q_X band of BChla is symmetrical and has a maximum at 608 nm, and no changes were observed during titration with TBAAc (or THEED) in the presence/absence of py (not shown). The lack of any effect of TBAAc/THEED on the spectrum of BChla in MeOH may suggest a relatively higher stability of the MeOH-ligated species compared with the [BChla(Ac)] complex. This seems, however, difficult to justify since the charge and chelating abilities would suggest an MeOH for Ac substitution. A straightforward possibility is that MeOH stabilizes Ac ions, for example, due to a strong hydrogen bonding.

These observations point to an easy substitution of two py ligands by one Ac at axial positions in BChla in MeCN. Consequently, a non-symmetrical complex in relation to the plane of the tetrapyrrole is formed. It is, however, unclear whether it is five- (tetragonal pyramidal) or six-coordinate (prismatic) since the spectral changes should be related to the influence of the axially coordinated ligands on the conformation of the macrocycle. Chelation is usually preferred over monodentate ligation. However, the four-membered rings are generally not as stable as five- or six-membered rings. Moreover, the proximity of the Chla π-electron system can, to some extent, counteract the formation of an additional Mg–Ac bond. Nevertheless, there is not enough space for any extra ligand and hence only one Ac can bind to the central Mg ion. As a result, the Mg ion chelated by Ac must be pulled out of the Chla plane more significantly than in the complex with a coordinated solvent molecule. Such displacement of the central ion causes some distortion of the tetrapyrrolic ring even though it is more rigid when compared to porphyrins.[32] The similar changes in the Q_X band which occur in the presence of high concentrations of THEED indicate that the effects of this chelator on BChla are comparable. However, they are weaker than the ones observed in the presence of TBAAc, so much so that a higher concentration of alcohol is required to substitute py in the [BChla(py)$_2$] complex.

The exceptionally strong binding of Ac to the central Mg ion and the deformation of the Chl macrocycle is fully confirmed by the absorption measurements under high pressure and the effects of acetate on the excited state lifetime of Chla and BChla and its demetalated derivatives. In the absence of Ac, the application of hydrostatic pressure at room temperature significantly shifts the coordination equilibrium of BChla in DMF toward the six-coordinate form (Figure 1C), whereas the addition of Ac (1 mM) very effectively stabilizes the five-coordinate state (Figure 1D). This effect is also reflected in the properties of the singlet excited state of the pigments; the addition of Ac to Chla solution in DMF causes a shortening of its fluorescence lifetime from 6.02 ± 0.06 to 5.85 ± 0.06 ns and to BChla in DMF, from 3.46 ± 0.07 to 3.14 ± 0.08 ns. At the same time, Ac has no effect on the fluorescence lifetimes of the demetalated pigments. As in other porphyrinoids, the shortening of the singlet excited state lifetime is an indication of the loss of planarity of the macrocycle.[32,33] Very similar effects on the coordinative and the excited state properties of the pigment were caused by intramolecular chelation of the central Mg in BChla (Fiedor et al., unpublished results).

Activation of copper(II): According to the available data, the Cu^{II} ion forms typical octahedral six-coordinate complexes in MeOH[34] and five-coordinate[35] or six-coordinate axially elongated octahedrons in MeCN.[36] Ac can obviously coordinate to the Cu^{II} ion, and in this process the coordinated solvent molecules (Solv) are substituted by Ac as its concentration increases. The speciation of Cu^{II} in the used solvents should be considered, especially since Ac can act either as a mono- or a bidentate ligand. X-ray studies performed by van Niekerk and Schoening[37] indicated that $CuAc_2{\cdot}H_2O$ crystallizes with Cu^{II} ions arranged in pairs which are bridged by four acetic groups. Moreover, $CuAc_2$ is supposed to form mainly dimeric complexes in various solvents, including acetic acid,[38–42] alcohols,[42,43] and MeCN.[44,45] The dimer to monomer dissociation constant was found to be very low in acetic acid, about 1×10^{-5} M, and increased up to 6×10^{-3} M in the order HAc < EtOH < MeOH.[42] Equilibrium (2) is strongly shifted towards the formation of the 1:2 complex:

$$[CuAc(Solv)_4]^+ + Ac^- \rightleftharpoons [CuAc_2Solv_2] + 2\ Solv \qquad (2)$$

A further increase in Ac concentration usually results in the formation of dimers, $[Cu_2Ac_4Solv_2]$. The monomeric species, $[CuAc_2Solv_2]$, is a tetragonally distorted octahedron with Ac more strongly bound to the Cu^{II} ion than the axial solvent molecules.[40] In the case of the dimer, one solvent molecule per Cu^{II} is released to enable a direct copper-to-copper bond formation.[37,46]

In order to determine the speciation in MeOH and MeCN, spectrophotometric titrations of trifluoromethanesulfonate ($CuTf_2$) with TBAAc were performed in both solvents. When the ratio of concentrations of Ac and Cu^{II}, $c_{Ac}/c_{Cu^{II}}$, was kept below 2, in both solvents similar changes could be seen in the absorption spectrum of $CuTf_2$ (Figure 3). The bands at 825 nm (in MeOH) and at 860 nm (in MeCN) disappeared while new bands accordingly appeared at 700 and 675 nm. The absorbance changes, monitored at 700 nm (or 675 nm), accompanying the increase in TBAAc concentration in the two solvents are shown in Figure 4A. At higher ratios of $c_{Ac}/c_{Cu^{II}}$, the changes of A_{700} in MeOH reach a plateau, whereas in MeCN a shift of the band maximum from 675 to 725 nm occurs and the band intensity decreases until $c_{Ac}/c_{Cu^{II}}=3$ is reached. An increase in the concentration of Ac also affects the higher energy band with a maximum near 325 nm.

The intensity of this band initially increases to reach a maximum at the ratio $c_{Ac}/c_{Cu^{II}}$ equal 2 in MeOH and equal 1

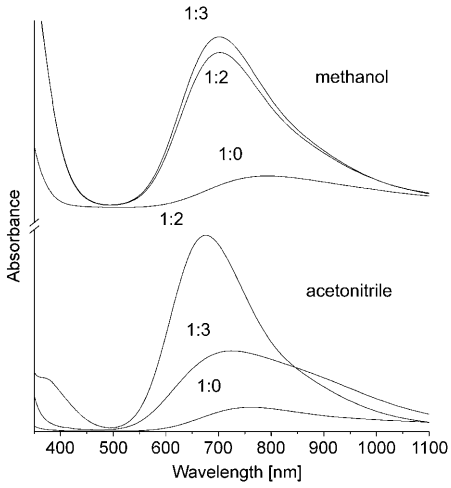

Figure 3. Electronic absorption spectra of the coordination forms of the Cu^{II} species in methanol and acetonitrile formed in the presence of various concentrations of tetrabutylammonium acetate. The numbers indicate the molar ratios between Cu^{II} ions and acetate.

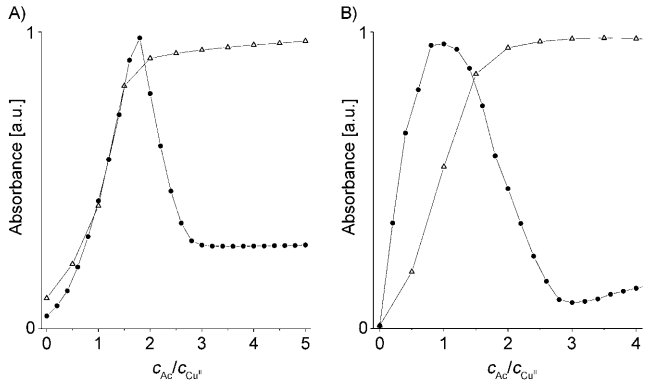

Figure 4. Titration of Cu^{II} trifluoromethanesulfonate ($CuTf_2$) with TBAAc in methanol (△) and acetonitrile (●) followed spectrophotometrically in the visible (A) and in the UV range (B). The absorbance changes in methanol were recorded at 700 nm and in acetonitrile at 675 nm. In the UV range, in both solvents the absorbance was followed at 375 nm. The signals were normalized and corrected for dilution (see text for details).

in MeCN. In the latter solvent, the A_{325} value drops to approximately half of its maximal value (at $c_{Ac}/c_{Cu^{II}}=2$) and remains unaffected by any further addition of TBAAc. Moreover, due to the narrowing of this band, another one can be observed at 375 nm which then decreases with increases in TBAAc concentration, disappearing completely when $c_{Ac}/c_{Cu^{II}}=3$. The titration curves plotted for absorbance changes at 375 nm are shown in Figure 4B. Due to a stronger overlap, the corresponding high energy band could not be distinguished in the spectra recorded in MeOH. However, in this solvent absorbance at 375 nm changed in proportion to the absorbance of the main charge transfer band at 325 nm.

Additional information was derived from EPR measurements. The spectra of $CuAc_2{\cdot}H_2O$ were recorded in MeOH and in MeCN, both in the absence and presence of 0.1 M TBAAc. In each case, a single signal was found with a maximum at about 3310G (see Supporting Information). The basic parameters of the signal are comparable with those reported by Grasdalen.[40–42] The most important change involves the baseline which under the influence of TBAAc remains wavy in MeOH over a wide field range, but straightens out in MeCN.

According to previous UV/Vis spectroscopic studies in various solvents,[39,42,47] the $[Cu_2Ac_4Solv_2]$ dimers show a considerably stronger absorption than $[CuAc_2Solv_2]$ and even stronger than $[CuAcSolv_4]^+$. In addition, the absorption band of the dimer is shifted towards shorter wavelengths compared to the monomer. Therefore, the spectral changes observed during the titration of $CuTf_2$ with TBAAc in MeOH point to both the coordination of two Acs as well as to the formation of $[Cu_2Ac_4(MeOH)_2]$. According to the earlier work of Tsuchida and Yamada,[43] and of Martin and Whitley,[47] the band at 375 nm should be considered diagnostic for dimeric Cu^{II} complexes. Thus, strong absorption signals at 700 and 375 nm indicate that Ac stabilizes such dimers in MeOH. Surprisingly, $[Cu_2Ac_4Solv_2]$ turned out to be unstable in MeCN in the presence of high TBAAc concentrations as indicated by a decrease in absorption at 375 and 675 nm, and a red shift of the latter band. This is confirmed by the EPR spectra. Since the resonance of the dimers is several thousand Gauss wide,[40,41] quantitative analysis to determine the concentration of $[Cu_2Ac_4Solv_2]$ was not possible. However, its concentration could be assessed from the decrease in intensity of the sharp signal characteristic of monomers observed at about 3300G in MeCN. In MeOH, the addition of Ac had no effect on the intensity of the monomer signal and the wavy shape of the line suggests an overlap with a very broad signal, which can be attributed to the contribution of dimers. Some traces of such a wave can also be recognized in the EPR spectrum of $CuAc_2$ in MeCN. Its disappearance upon the addition of TBAAc indicates decomposition of the dimers.

The different behavior of copper dimeric species in MeOH remains unresolved. One should consider the possibility of a proton release by MeOH molecules caused by the influence of the Cu^{II} ion on their pK_a value. Furthermore, this proton release could be assisted by Ac as Cu^{II} methoxide can be easily formed in MeOH.[48] The existence of $[Cu_2Ac_4(MeO)_2]^{2-}$ provides another justification for its high stability in the presence of TBAAc.

The results discussed above confirm that $CuAc_2$ in MeOH and MeCN exists mainly in dimeric forms, although monomerization can occur as a result of the coordination of the third Ac in MeCN. The stability of chelates in the $[CuAc_3]^-$ complex must be even lower than in $[CuAc_2Solv_2]$, such that further Ac substitution can occur quite easily. No evidence for the $[CuAc_4]^{2-}$, $[CuAc_5]^{3-}$ or $[CuAc_6]^{4-}$ forms was obtained from spectroscopic studies. Thus, we assume that in an excess of Ac the predominant species of Cu^{II} in MeCN is $[CuAc_3]^-$, whereas in MeOH it is $[Cu_2Ac_4(MeOH)_2]$ or $[Cu_2Ac_4(MeO)_2]^{2-}$.

A THEED-induced decomposition of dimeric Cu^{II} species is not expected as the electron-donating abilities of hydroxy groups are not sufficient to overcome the coordination of Ac. Therefore, substitution of MeCN in $[Cu_2Ac_4Solv_2]$ by THEED does not affect the overall complex structure. Some additional interactions can be considered, especially hydrogen bond formation with bridging Ac oxygen atoms. Nevertheless, their positive influence on Cu^{II} activity is questionable.

Activation of chlorophyll: The effect of Ac on both the Cu^{II} ion and the tetrapyrrolic system, suggests possible implications for the metal exchange reactions of Chla. A series of kinetic studies with Chls and $CuAc_2$ were performed by Berezin et al.,[49,50] but the influence of the monomer–dimer equilibrium on the kinetics of the metalation of pheophytin (free base, Pheo) was not discussed in any further detail. Therefore, the reactions of Chla and, in addition, Pheo with $CuAc_2$ were studied in MeOH and MeCN, both in the absence and presence of TBAAc and THEED. The course of these reactions was followed using absorption and emission (fluorescence) spectroscopy.

In both solvents at ambient temperature, the substitution of Mg by Cu^{II} in Chla occurred relatively slowly as only small changes were observed in the absorption spectra (not shown) of the reaction mixtures containing 2.5 μM Chla and a large excess of the Cu^{II} salt (5 mM in MeCN and 2.5 mM in MeOH). In MeOH, a blue shift of a few nanometers of the Q_Y band of Chla occurred, but the reaction did not proceed further as no shift of this band to 642 nm (characteristic of the Cu–Chla product) was observed. Through prolonged periods of time, a slow degradation of Chla was observed in MeCN (not shown). The reactions of $CuAc_2$ with Chla were repeated in the presence of TBAAc and THEED.

As shown in Figures 5 and 6, a strong effect of TBAAc on the transmetalation reaction was observed in MeCN. TBAAc caused rapid changes in both the electronic absorption and emission spectra of the reaction mixture. In the absorption spectrum, a fast decrease of the band at 662 nm (Chla) occurred, accompanied by an increase of the band at 642 nm (Cu–Chla) and a change of the shape of the Soret band (Figure 5). The appearance of at least two isosbestic points (near 430 and 655 nm) suggests that no intermediates occur during the formation of Cu-substituted Chla. The reaction seems to be irreversible, which is consistent with the finding that the observed absorbance changes both at 642 and 662 nm are independent of the Cu^{II} concentration (not shown).

Some acceleration of the transmetalation reaction was also caused by the addition of THEED, but this effect was less pronounced than in the case of TBAAc (see Supporting Information). Also, THEED caused a smaller shift of the Q_Y band of Chla, in this case to 650 nm. It follows then that THEED as a chelator shows weaker coordinative properties towards the central Mg^{2+} ion as compared to Ac. Seemingly, THEED somewhat distorts the conformation of Chla and thus facilitates an interaction with the Cu^{II} ion on the opposite side of the macrocycle. Although this deformation and the Mg–N bond stretching may not be sufficient for a dissociation of the central Mg^{2+}, a bimetallic intermediate complex, Mg-Chla-Cu, can presumably be stabilized.

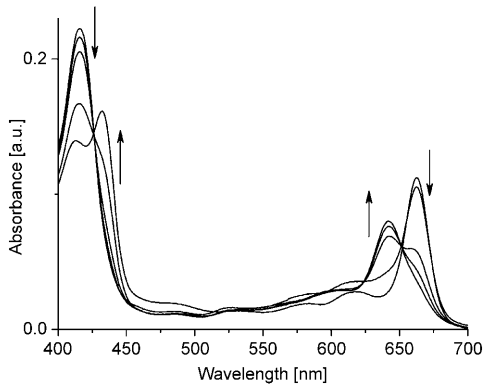

Figure 5. Spectral changes accompanying the acetate-assisted transmetalation of chlorophyll a with Cu^{II} acetate in acetonitrile (see text for details).

The kinetics of the reactions in MeCN in the presence of 0.1 M TBAAc and 0.1 M THEED, followed at 642 nm, are compared in Figure 6, together with the kinetics of the reaction of Chla with $CuAc_2$ performed in the absence of any additives (lower trace). Clearly, both chelators cause a substantial increase in the rate of the transmetalation reaction. In MeOH, Ac had inhibiting effects on the transmetalation reaction (not shown).

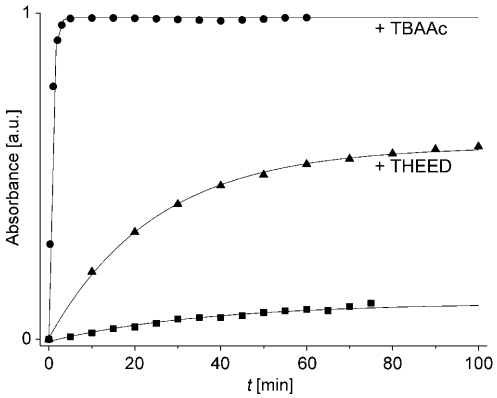

Figure 6. Effects of TBAAc and THEED on the kinetics of conversion of chlorophyll a into the copper complex (see text for details). The transmetalation was carried out with $CuAc_2$ alone (■) and with the addition of 0.1 M THEED (▲) and 0.1 M TBAAc (●). The reaction progress was monitored at 642 nm. Solid lines represent curve fits to the experimental points.

Figure 7 shows the fluorescence changes that accompany the reaction of Chla with $CuAc_2$ in MeCN in the presence of 0.1 M TBAAc. They are representative of all the reactions studied since a two-step decrease in the emission of Chla was found in each case. The first step, during which the fluorescence intensity decreased by 70–90%, reaching comple-

tion within the mixing time of the reactants in the cuvette, turned out to be too fast to be followed using a conventional spectrofluorometer. The next step involved a further but much slower decrease in emission intensity and was quite similar in all transmetalation reactions.

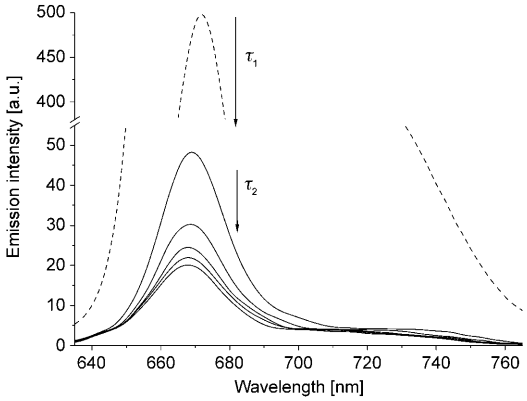

Figure 7. Decay of chlorophyll a fluorescence occurring in the presence of Cu^{II} and TBAAc in acetonitrile at 298 K. In the first rapid phase, the emission intensity decreased by almost 90%, and then a slower phase followed (see text for details).

Figure 8 shows the kinetics of the decay of chlorophyll fluorescence, observed in the presence of different concentrations of TBAAc. The decay curve is zero-order when the concentration of Ac is less than three times the concentration of the Cu^{II} ions. This should be related to the very slow decrease in the intensity of the absorption bands of Chla which occurs in the absence of TBAAc (not shown). At higher concentrations of Ac ($c_{Ac}/c_{Cu^{II}} > 3$), fluorescence decay can be fitted with a single exponential function, thus indicating a change in the order of the reaction. The reaction rates increase further with increasing Ac concentration.

On the basis of these observations, the acceleration of Chla transmetalation should be ascribed mainly to the presence of ligands (L) that can pull the central Mg^{2+} ion out of the Chl plane, with the rate and equilibrium constants of the metal exchange reactions dependent on the relative stabilities and structures of the corresponding Mg-Chla-L species. This effect should be particularly distinct with chelators because ring-closure in the Chl–L complex usually lengthens the Mg–N bonds or, in some cases, vacates one of the pyrrolenine nitrogens. For this reason, the presence of Ac would not be expected to affect the reaction of free base metalation. In order to check this assumption, the reactions of Pheo and $CuAc_2$ were also studied both in the absence and presence of TBAAc. No significant influence of Ac on the kinetics of the metalation reaction was found, either in MeOH or in MeCN (data not shown).

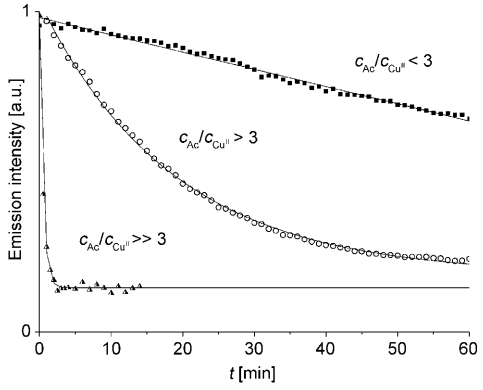

Figure 8. Dependence of the chlorophyll a fluorescence decay profile on the relative ratio of concentrations of Cu^{II} and acetate (as TBAAc). Solid lines represent curve fits to the experimental points.

Apparently, the influence of Ac on the transmetalation reaction of Chla can be controlled by the conformation of some peripheral substituents of tetrapyrrole. As shown by many authors,[51–56] the isocyclic ring V is of great importance for the coordination properties of the Chla core. Due to the presence of enolisable β-ketoester at ring V (Figure 1), Chls may occur in either keto or enol form. The double bond in the isocyclic ring, occurring in the enol form, draws electrons from the tetrapyrrole system and causes a meaningful weakening of the Mg–N bonds.[54] Enolysation would be then restrained when the proton at carbon C-13^2 is substituted by for example, a hydroxyl group. Another way to prevent enolysis of ring V is the removal of the methoxy carbonyl group (e.g. via pyrolysis), which is a component of the β-ketoester.[51] Therefore, Chla and two of its derivatives, 13^2-hydroxychlorophyll a (Chla-OH) and 3-acetyl-pyroChla, were compared in terms of their reactivity in metal exchange reactions in MeCN. The reaction conditions were identical to those used for Ac-assisted Chla transmetalation, that is, 0.1 M TBAAc, 2.5 μM Chla-OH, 2.5 μM 3-acetyl-pyroChla, $T = 298$ K. Transmetalation was not observed within the range of 0.5–5 mM $CuAc_2$ either with Chla-OH or with 3-acetyl-pyroChla, thus indicating that the effect of Ac applies first and foremost to the activation of the tetrapyrrole ring. Since neither Chla-OH nor 3-acetyl-pyro-Chla undergo metal substitution in the presence of high concentrations of Ac, one can conclude that the influence of keto form stabilization on the strength of Mg–N bonds prevails over the labilizing effect of Ac.

Kinetic studies: A detailed kinetic study was performed on Ac-induced Chla transmetalation in MeCN. The reaction was investigated independently for a fixed concentration of either Cu^{II} or Ac. As shown in Figure 9A, the observed rate constants increased linearly with increasing Ac concentration for $c_{Ac}/c_{Cu} > 3$ and the reaction followed rate Equation (3):

$$k_{obs} = k_a c_{Ac} \quad (3)$$

for which the second-order rate constant at 298 K was $0.152 \pm 0.006\ M^{-1}s^{-1}$. Regardless of the temperature, no meaningful reaction occurred below a total Ac concentration of 15 mM, which corresponds to $c_{Ac}/c_{Cu^{II}} = 3$. This finding is in agreement with the results of the spectroscopic investi-

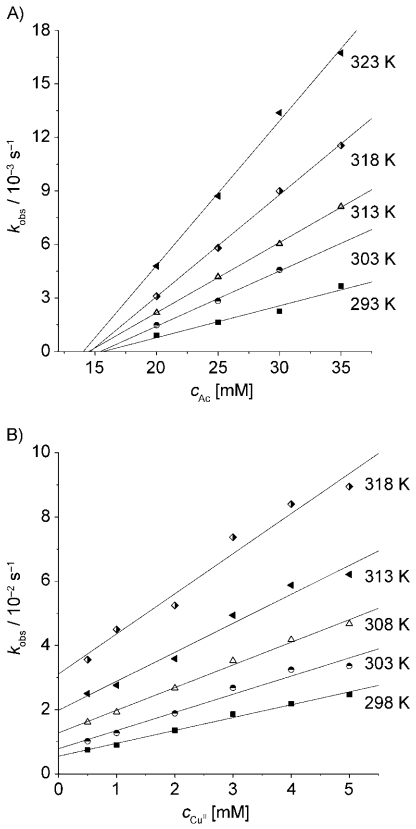

Figure 9. Dependence of rate constants of the acetate-assisted transmetalation of chlorophyll a with Cu^{II} acetate, carried out in acetonitrile, on the concentration of A) added acetate (as TBAAc) at a fixed concentration of Cu^{II} ions, and B) Cu^{II} ions at a fixed concentration of acetate. The transmetalation was monitored spectrophotometrically at 640 nm.

gation discussed above, which showed that in MeCN the monomeric Cu^{II} species predominates at least three-fold over Ac. In contrast to dimeric $[Cu_2Ac_4Solv_2]$ species, $[CuAc_3]^-$ monomers can easily exchange Ac for tetrapyrrole, and thus enable the formation of a Cu–Chla bond. At a fixed Ac concentration (0.1 M), the rate constant increased with increasing Cu^{II} concentration (see Figure 9B). The linear dependence of k_{obs} on c_{Cu} showed a clear intercept such that the rate expression was given by Equation (4).

$$k_{obs} = k_b c_{Cu^{II}} + k_c \quad (4)$$

At 298 K and $c_{Ac} = 0.1$ M, $k_b = 4.0 \pm 0.2\ M^{-1} s^{-1}$ and $k_c = (5.5 \pm 0.6) \times 10^{-3}\ s^{-1}$. Summarizing Equations (3) and (4), the overall rate expression can be given by Equation (5).

$$k_{obs} = k_1 c_{Cu^{II}} c_{Ac} + k_2 \quad (5)$$

The k_1 value was calculated from the values of k_a and k_b by taking $c_{Cu^{II}}$ and c_{Ac} into account, respectively. The values of k_1 fall indeed very close to an average value of $35 \pm 5\ M^{-2} s^{-1}$ at 298 K (Table 1).

Activation parameters were derived from both concentration dependencies. At a fixed Cu^{II} concentration, activation

Table 1. The rates and activation parameters for the transmetalation of chlorophyll a with Cu^{II}. The kinetics controlled by the concentration of Cu^{II} ions was studied in the presence of 0.1 M acetate, and the kinetics controlled by the concentration of acetate was studied in the presence of 5 mM Cu^{II}.[a]

Rate constants	k_{298}	$\Delta H^{\neq}$ [kJ mol^{-1}]	$\Delta S^{\neq}$ [J mol^{-1} K^{-1}]
k_a, $c_{Cu^{II}}$ const.	$(15.2 \pm 0.6) \times 10^{-2}\ M^{-1} s^{-1}$	43 ± 2	-115 ± 9
k_b, c_{Ac} const.	$4.0 \pm 0.2\ M^{-1} s^{-1}$	41 ± 2	-97 ± 6
$k_c = k_2$, c_{Ac} const.	$(5.5 \pm 0.6) \times 10^{-3}\ s^{-1}$	67 ± 2	-64 ± 4
$k_1 = k_a / c_{Cu^{II}}$	$30.4 \pm 1.2\ M^{-2} s^{-1}$	43 ± 2	-72 ± 4
$k_1 = k_b / c_{Ac}$	$40.0 \pm 2.0\ M^{-2} s^{-1}$	41 ± 2	-78 ± 4

[a] The symbols used in the table refer to the Equations (3–5). Experimental procedures are described in the Materials and Methods Section.

entropy and enthalpy were found to be $+43 \pm 2$ kJ mol^{-1} and $+118 \pm 10$ J mol^{-1} K^{-1}, respectively (see Table 1). The clear intercept found for the dependence of k_{obs} on $c_{Cu^{II}}$ allowed the determination of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ individually for the Cu-dependent and the Cu-independent pathways (Table 1).

As shown by the dependence of k_{obs} on Ac concentration, the rate of Chla transmetalation in MeCN increased with increasing Ac concentration up to a large excess over $c_{Cu^{II}}$ and c_{Chla}. Since the addition of TBAAc activates both Cu^{II} and Chla, the equilibria given by Equations (6) and (7) should be taken into consideration.

$$[Cu_2Ac_4Solv_2] + Ac^- \rightleftharpoons [CuAc_3]^- + [CuAc_2Solv_2] \quad (6)$$

$$\text{Mg-Chla} + Ac^- \rightleftharpoons \text{Ac-Mg-Chla} \quad (7)$$

Earlier work has shown that Cu^{II} dimers are stable enough to prevent the Cu^{II} ion from undergoing ligand exchange, in particular for tetrapyrrolic systems.[57] Therefore, Equation (6) involves the monomerization of the Ac-bridged Cu^{II} complex. Such a process requires that Ac ions outnumber Cu^{II} ions by more than 2 to 1. One can assume that an increase in Ac concentration so that there is at least a two-fold excess of Ac over Cu^{II}, would result first of all in coordination of the Mg ion as the solvent molecules are substituted in the axial position of Chla, by which the macrocycle is activated. Then the displacement of solvent molecules by Ac ions in dimeric Cu^{II} complexes would cause a considerable strain that leads to a breakage of the bridging Cu–O bonds. Ac ions can act both as mono- and bidentate ligands in monomeric complexes of Cu^{II}. Since the small four-membered rings are not favored due to a considerable steric strain,[41] one can assume a labilization of the Cu–O bonds and the facilitation of ligand exchange. In turn, the coordination of the Cu^{II} ion by tetrapyrrole is facilitated.

Kinetic measurements both at constant Cu^{II} and Ac concentrations, showed that the enthalpy of activation for k_a and k_b is very similar regardless of the way it was determined and has an average value of 42 ± 3 kJ mol^{-1}. Such conformity in the $\Delta H^{\neq}$ values is in agreement with what is predicted by Equation (5). Taking the concentrations of Cu^{II}

(3) and Ac (4) into account does not affect the enthalpy values obtained for the third-order rate constant k_1. The lack of activation parameters in the literature for Chla transmetalation does not allow a direct comparison, although the value is quite typical for the incorporation of Cu^{II} ions into tetrapyrrolic systems.[58–61] Since some distortion of the porphyrin from planarity is required for further metal coordination, the values of $\Delta H^{\neq}$ are usually related to the energy required for such a deformation. However, in the case of Ac-assisted transmetalation, the macrocycle bends when the Mg ion is pulled out of the plane so that the relatively high rigidity of the chlorophyll ring is balanced by the effect of the axial ligand.

The values of the activation entropy, obtained from the dependence of the second-order rate constants on temperature, are both negative and moderately large. However, the actual $\Delta S^{\neq}$ for the overall reaction was calculated from the dependence of the third-order rate constants on temperature, at fixed concentrations of Cu^{II} and Ac. The obtained values, namely -72 ± 4 and $-78\pm4\,J\,K^{-1}mol^{-1}$, are very close to each other and in line with what is expected from Equation (5). Finally, the average activation entropy for k_1 was found to be $-75\pm7\,J\,K^{-1}mol^{-1}$. This relatively large and negative $\Delta S^{\neq}$ value may indicate an associative type of mechanism. The formation of the first bond between the tetrapyrrolic ligand and the incoming metal ion is usually recognized as the rate-limiting step for both metalation and transmetalation of porphyrins. In the case of metal exchange in Chla it requires a movement of the Mg^{2+} ion. Ac coordination orders the system and is accompanied by a significant lengthening of the Mg–N bond. Moreover, the Cu^{II} ion probably does not require the release of any ligand at this stage because the Ac ions dissociate only from the binuclear Mg-Chla-Cu-Ac species. These bond-formation contributions must account for the significantly negative activation entropy.

The first-order rate constants k_c (4) and k_2 (5) can either be due to a reverse or a parallel reaction that involves a Cu-independent rate-limiting step. The independence of the overall absorbance change observed during the reaction on the Cu^{II} concentration rules out any possible contribution from a reverse reaction. Hence, it is reasonable to attribute k_2 (5) to some Chla transformation that involves an Ac-induced tetrapyrrole deformation. As a result, a more reactive Chla species is formed which reacts rapidly with a Cu^{II} ion in a subsequent step. Another plausible interpretation involves Ac dissociation from the Cu^{II} complex prior to coordination to the pyrrolenine nitrogen. This, however, seems unlikely because the Cu^{II} equilibria should then affect the Cu-independent pathway.

The transmetalation reactions in MeOH followed a very different pattern. The kinetics of this process were measured at constant $c_{Cu^{II}}$ and varied c_{Ac}, and the determined k_{obs} values were plotted against the $c_{Ac}/c_{Cu^{II}}$ ratio (Figure 10). At a low concentration of Ac the kinetic traces had a complex zero–-first-order character and the contribution of the linear decay of the absorbance at 665 nm decreased with increasing concentration of added TBAAc. This corresponds to the substitution of axially coordinated solvent molecules by the first Ac ligand. Moreover, the k_{obs} values for the metal substitution process decreased as $[CuAc_2(MeOH)_2]$ and $[Cu_2Ac_4(MeOH)_2]$ became the dominating species in the reaction mixture, approaching zero at a great excess of Ac over $c_{Cu^{II}}$. Since Cu^{II} mainly occurs as a dimer under such conditions, $[Cu_2Ac_4(MeOH)_2]$ turned out to be inert and $[CuAc(MeOH)_4]^+$ to be the most reactive species in the transmetalation of Chla.

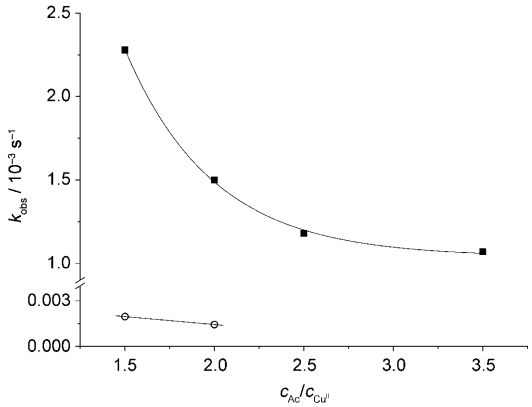

Figure 10. Dependence of rate constants (k_{obs}) of two competing reactions, oxidation (○) and transmetalation (■), of chlorophyll a with the Cu^{II} complexes in methanol on the concentration of added acetate. Kinetics were determined from spectrophotometric monitoring of the reactions at 665 nm.

Mechanistic interpretations: The exchange of Mg by Cu^{II} in Chla is usually a slow reaction which lasts several hours under standard conditions, especially when a Cu^{II} ion with a strongly coordinating ligand is used. The choice of the ligand is, however, essential from a mechanistic point of view since it tunes the redox potential of the coordinated metal ion. This is of significance in the case of the Cu^{II} ion which, under some conditions, can oxidize tetrapyrrole to its cation radical. Ac secures non-redox pathways for the formation reaction of a Cu derivative of Chla and therefore, $CuAc_2$ was used as the reactant.

The catalytic effect of carboxylate anions on Cu^{II} ion incorporation into tetrapyrrole has been previously observed by Johnson et al.[62] and by Sugata and Matsushima.[63] Both studies showed that small amounts of Ac can significantly accelerate the reaction of the Cu^{II} ion with a porphyrin free base. The observed rate constants reached a maximum when the concentrations of Ac and Cu^{II} were comparable, thereby suggesting that $[CuAc(Solv)_4]^+$ is the most reactive species in the system. This conclusion agreed with the results obtained by other authors from kinetic studies on porphyrin metallation in acetic acid.[64,65] Whereas the formation of non-reactive dimers could be expected at a higher Ac concentration, activation of fully solvated Cu^{II} species, which occurs via solvent–ligand exchange for Ac, may seem surprising to some extent. This effect can, however, be justified

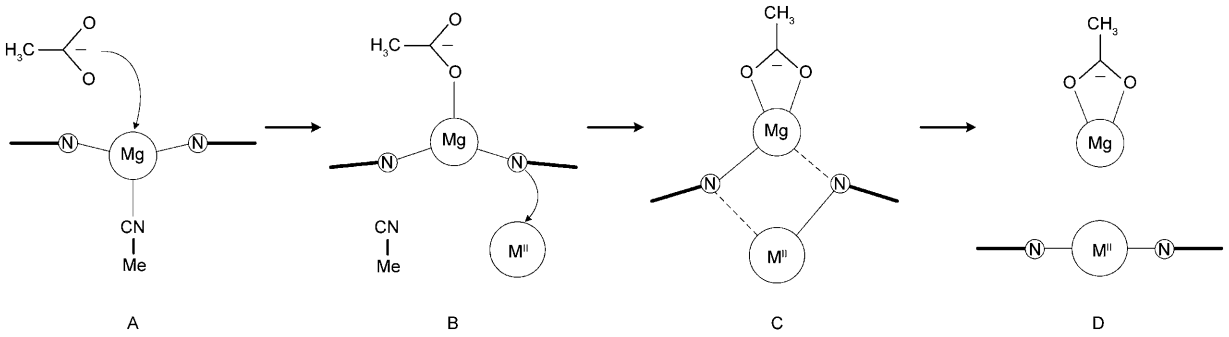

Figure 11. Proposed steps of acetate-assisted transmetalation of chlorophyll.

by the facilitation of proton release from the porphyrin core.

Similar experiments with Chls turned out to be impossible because the use of Cu^{II} salts with non-coordinating counter ions may lead to the oxidative degradation of the pigment. Transmetalation of Chla with $CuAc_2$ does occur, albeit very slowly, especially in MeCN, where direct metal substitution seems to compete with the formation of a stable complex between Cu^{II} ion and Chla, probably of a charge-transfer character, as indicated by a strong quenching of Chla fluorescence in the presence of Cu^{II} ions (see below) and the appearance of a broad absorption band above 800 nm (not shown). This points to the presence of some traces of solvated Cu^{II} ions. Therefore, one should expect the other species with mixed ligands, such as $[CuAc(Solv)_4]^+$ and $[CuAc_2Solv_2]$, to be present in an MeCN solution of $CuAc_2$, although they cannot react with Chla unless it is activated.

Although a similar speciation is expected for $CuAc_2$ dissolved in MeOH, the transmetalation reaction occurs only to some extent as indicated by the blue shift of the Chla Q_Y band. The reason why Chla undergoes metal exchange with $CuAc_2$ in MeOH but not in MeCN, is presently not so clear. One possibility is that hydrogen bonding between MeOH and Ac can lead to a lower "effective" concentration of Ac and thereby to a higher concentration of Cu^{II} monomers. Another explanation might be the deprotonation of coordinated MeOH and thus a stabilization of solvated Cu^{II} species in the methoxy form.

The situation changes drastically in the presence of higher Ac concentrations. Such conditions favor transmetalation of Chla in MeCN. Since no effect was found for Pheo metalation, one can assume that the facilitation of pyrrolenine proton release cannot significantly control metal incorporation in this case. The further decay of dimers in MeCN did not affect the rate of Cu^{II} ion insertion, indicating the crucial role of the central Mg ion in Chla activation.

Fluorescence measurements showed that, regardless of the presence of Ac, some interaction of the Cu^{II} ion and Chla occurs in the very first stage of the reaction. This should be attributed to the formation of an outer-sphere complex rather than to the actual coordination of the Cu^{II} ion. Such electrostatic interactions are known to initiate the conformational changes in porphyrins, which in turn are crucial for subsequent complexation steps.[35,66–71] Some authors suggest a reverse order of the first steps in which outer-sphere complex formation results from an earlier deformation of the macrocycle. It seems reasonable that initial ring distortion favors electrostatic interaction, which facilitates further deformation. The out-of-plane position of the central Mg ion in MeCN (Figure 11, structure A) justifies an outer-sphere complexation of the Cu^{II} ion. It is, however, insufficient to increase distortion in the macrocycle especially as the isocyclic ring renders it more rigid compared to porphyrins. Hence, the exchange of the Mg axial ligand by more bulky and charged ligands can induce the reaction (Figure 11, structure B). It should be even more distinct for chelating ligands as ring-closure lengthens the Mg–Chla bonds.

Pulling the Mg ion out of the Chla plane is accompanied by the first Cu–N bond formation step that leads to the formation of a bimetallic complex in which Cu^{II} and Mg are bound to opposite sides of the tetrapyrrolic ligand (structure C). Such types of intermediates have been considered in the mechanism of porphyrin transmetalation.[72,73] The subsequent dissociation of the Mg ion from Chla permits the chelation of Cu^{II} and, as a consequence, the release of solvent and Ac ligands.

The mechanism of the acetate-assisted transmetalation of Chl mimics in a way the sequence of steps which occur during biological insertion of metal ions into porphyrins. Most-studied is the insertion of the Fe^{2+} ion into protoporphyrin IX to yield heme, catalyzed by ferrochelatases. Recent crystallographic investigations reveal the presence of conserved acidic residues (glutamate and aspartate) in the metal biding sites on the enzymes, obviously acting as chelating groups, similarly to the role played by acetate in the transmetalation. On the other hand, the inhibitory studies, the use of catalytic antibodies and crystallographic investigations all reveal that out-of-plane distortion of porphyrin is an essential step in the biological catalysis. Furthermore, the metalation is strongly enhanced in distorted (e.g. N-substituted) porphyrins.[74] The exact mode of this distortion (domed, ruffled or saddled) is still debated but it is agreed that the strain exposes the nitrogen atoms of the porphyrin

macrocycle.[75,76] This strained structure resembles the bimetallic species (structure C) in the proposed mechanism of Chl transmetalation.

Conclusion

Mg for Cu^{II} exchange in Chla is significantly accelerated by acetate, whose role is two-fold: activation of both Chla and the Cu^{II} ion. The most important effect consists of an axial coordination to Chla, which leads to the pulling of the central Mg ion out of the macrocyclic plane. The out-of-plane displacement of the central ion makes the tetrapyrrolic system more susceptible to electrophilic attack. Moreover, the susceptibility of the core nitrogens to electrophilic attack is in part controlled by the enolisable system at isocyclic ring V. The influence of acetate on the reactivity of Cu^{II} depends on the properties of the solvent, which can either stabilize dimeric species or facilitate their dissociation to reactive monomers. It is especially distinct if protic and aprotic solvents are compared because proton release from the coordinated molecule stabilizes the metal-solvent bond and thus prevents substitution by acetate. On the other hand, protic solvents deactivate acetate ions due to hydrogen bonding. Furthermore, the differences in solvent coordination to central Mg ion in Chl entail differences in the geometry of the macrocycle and, as a result, differences in Chl ability for ligand exchange and further deformation.

These new insights into the mechanism of the acetate-induced transmetalation of Chls reveal novel aspects of the interplay between the peripheral groups and the reactivity of the core nitrogens. These results point to key differences between chlorophylls and less derivatized porphyrins in interactions with metal centers. Viewed from a broader perspective, they can also serve as guidelines towards the control of the chemical reactivity of tetrapyrroles and for the design of more efficient methods of preparation of metallosubstituted porphyrins and related molecules.

Experimental Section

Pigment preparation: Chla was extracted from cyanobacterium *Spirulina laporte* using the method described by Iriyama et al.[77] It was purified on a DEAE Sepharose column and then by isocratic HPLC on a semi-preparative reversed phase (C-18) column (Varian, 250×4.6 mm), using neat methanol (MeOH) as the eluent (flow rate 4 mL min^{-1}).[78] BChla was isolated from a purple bacterium *Rhodobacter sphaeroides* following Omata and Murata.[79] It was purified by column chromatography on DEAE Sepharose using 15% MeOH in acetone as the eluent. The pure pigments were dried under vacuum and stored at 244 K under Ar. Other chlorophyll derivatives, 13^2-hydroxychlorophyll a and 3-acetylpyrochlorophyll a, were a generous gift from Prof. Hugo Scheer (University of Munich). The solutions of pigments in acetonitrile (MeCN) and MeOH were freshly prepared before use and their concentrations were calculated on the basis of the respective extinction coefficients.[80]

Kinetic measurements: All kinetic measurements, including temperature studies, were carried out under pseudo-first-order conditions with at least a 200-fold excess of copper salt. The fast kinetics were measured on a stopped-flow apparatus (Applied Photophysics Ltd., Surrey, UK). The temperature was controlled with a Grant LTD6G thermostat. Kinetic measurements of slower reactions (at lower Ac concentration) were performed using a Perkin Elmer Lambda 35 spectrophotometer. The formation of Cu–Chla in the presence of TBAAc was monitored as an absorption increase at 640 nm. To determine the influence of the concentration of Cu^{II} on the kinetics of Cu–Chla formation, the concentration of $CuAc_2$ was varied in a range between 0.5 and 5 mM at a fixed concentration of TBAAc of 0.1 M. The influence of the Ac concentration was studied for solutions containing 5 mM $CuAc_2$ and from 0 to 25 mM TBAAc. The measurements were performed within a temperature range between 293–323 K in steps of 5 K.

Interaction between chlorophyll and acetate ions: In order to follow the interactions of Chls with potential axial ligands, the titrations of BChla (2.5 μM) in organic solvents were performed using py as a ligand, and TBAAc and THEED as chelators. To compare the strength of ligand binding to BChla, the pigment was either pre-incubated in 0.1 mM solutions of TBAAc or THEED and then titrated with py up to a concentration of 0.15 mM, or first pre-incubated with 0.15 mM py and then treated with TBAAc or THEED, both up to 0.2 mM. The changes in the coordination state of the pigment were monitored spectrophotometrically. The experiments were performed at ambient temperature.

Determination of the structure of Cu^{II} complexes in methanol and acetonitrile: The coordinative forms of Cu^{II} in MeOH were investigated by absorption spectroscopy at room temperature. A 2 mL sample of a 5 mM $CuTf_2$ solution was treated in a standard 1 cm cuvette with portions of TBAAc. The Ac concentration was increased in steps of 1 mM in MeCN and 2.5 mM in MeOH. The mixture was shaken and the spectra were recorded in the range of 350–1100 nm. The titration was continued as long as the changes in the absorption spectra were observable. An identical experiment was performed with $CuAc_2$ instead of $CuTf_2$. Additionally, the EPR spectra of $CuAc_2$ in MeOH and MeCN were recorded at 77 K, both in the absence and presence of 0.1 mM TBAAc, using a Bruker ELEXYS 500 instrument.

Transmetalation of chlorophyll a by Cu^{II} acetate in methanol: A solution of 5 μM Chla in MeOH and a solution of 5 mM $CuAc_2$ in MeOH were placed in a 0.88 cm tandem cuvette and the initial absorption spectrum was recorded within a range of 350–750 nm. The reagents were mixed and repetitive scan measurements at intervals of 60 s were performed in order to monitor the progress of the reaction. Ac-assisted transmetalation was investigated in the same way. In this case, both solid TBAAc and solid $CuAc_2{\cdot}H_2O$ were dissolved in MeOH to obtain concentrations of 0.2 and 0.01 M, respectively. Other concentrations of $CuAc_2$ used in kinetic studies were obtained by an appropriate dilution with 0.2 M TBAAc. The highest concentrations of Cu^{II} and Ac used in the reaction with Chla were 5 mM and 0.1 M, respectively. Fluorescence measurements for the Chla transmetalation in MeOH were carried out at 298 K with the excitation and emission slits being set at 15 and 5 nm, respectively. The solution of Chla was excited at 415 nm and the fluorescence spectra were recorded within the range of 600–800 nm.

Transmetalation of chlorophylls by Cu^{II} acetate in acetonitrile: The measurements performed during the reaction of Chla with $CuAc_2$ in MeCN were identical to those described for the reaction in methanol but a higher concentration of $CuAc_2$, that is, 10 mM, was applied. In preliminary experiments, sixty scans of absorption and emission spectra were recorded at an interval of 1 min both in the absence and presence of TBAAc. The kinetic measurements were conducted within a much narrower timescale, using either conventional electronic absorption spectroscopy or the stopped-flow technique. In order to determine the influence of the Ac concentration on the reaction rate, the concentration of TBAAc was varied within a range of 5 and 25 mM, whereas the concentrations of Chla and Cu^{II} were kept constant at 2.5 μM and 5 mM, respectively. Each concentration dependence measurement was performed at seven temperatures between 293 and 323 K in steps of 5 K. The k_{obs} dependence on the Cu^{II} concentration was studied at concentrations of Chla and TBAAc fixed at 2.5 μM and 0.1 M, respectively. The concentrations of Cu^{II} were the following: 0.5, 1, 2, 3, 4 and 5 mM. The measurements were repeated at various temperatures within a range of 298–318 K in steps of 5 K, at ambient pressure.

The influence of THEED on the transmetalation of Chla by $CuAc_2$ was studied at 298 K in the presence of 20, 50 and 100 mM THEED. The effect of TBAAc was also tested in the reactions of $CuAc_2$ with Chla derivatives. Similarly to the described transmetalation reactions of Chla, Chla-OH and 3-Ac-pyroChla were used at a concentration of approximately 2.5 μM. The concentrations of $CuAc_2$ and TBAAc were identical with those used in the reactions of unmodified Chla and the temperature was set at 298 K.

Reagents: Cu^{II} acetate monohydrate and TBAAc were obtained from Aldrich (Sigma-Aldrich, Germany). MeCN (Acros Organics, Belgium) was of chromatographic grade. MeOH and py (analytical grade) were purchased from POCh (Gliwice, Poland). THEED (analytical grade) was obtained from Fluka (Germany).

Spectroscopic measurements: Absorption spectra were recorded on a Perkin Elmer Lambda 35 spectrophotometer in 0.88 cm tandem cuvettes. Temperature was controlled with a PTP-6 Peltier system (Perkin Elmer). A $CuAc_2 \cdot H_2O$ solution (in MeOH or MeCN) was used as a reference in the spectroscopic studies. Fluorescence was measured in standard 1 cm four-window cuvettes with a Perkin Elmer LS55 spectrofluorometer equipped with a Grant LTD6G thermostat. Samples were excited at 415 nm and the emission was measured within a range of 600–800 nm.

Time-resolved fluorescence: The fluorescence lifetime measurements were performed using a K-2 multifrequency cross-correlation phase and modulation fluorometer (ISS Instruments, USA), as described previously.[81,82] The average random errors in the experimental data (fluorescence lifetimes) were evaluated automatically by the program used for data analysis on the basis of two parameters of emitted fluorescence: the phase shift and demodulation ratio with reference to the scattering of glycogen suspension in water.

High-pressure measurements: The effects of high hydrostatic pressure (up to 200 MPa) on the absorption spectra of BChla were measured on a custom-built high pressure probe, equipped with a Grant LTD6G thermostat, according to a previously described method.[83,84] The absorption spectra were measured using a Shimadzu 2101PC spectrophotometer.

Acknowledgements

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