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Steric Control of Bacteriochlorophyll Ligation

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Abstract: The axial coordination of central Mg^{2+} ion in chlorophylls is of great structural and functional importance for virtually all photosynthetic chlorophyll proteins; however, little thermodynamic data are available on the ligand binding to these pigments. In the present study, spectral deconvolution of the bacteriochlorophyll Q_X band serves to determine the ligand binding equilibria and relationships between thermodynamic parameters of ligand binding, ligand properties, and steric interactions occurring within the pigment. On the basis of the temperature effects on coordination, ΔH°, ΔS°, and ΔG° of binding various types of ligands (acetone, dimethylformamide, imidazole, and pyridine) to diastereoisomeric bacteriochlorophylls were derived from respective van't Hoff's plots. At ambient temperatures, only ligation by imidazole and pyridine occurs spontaneously while ΔG° becomes positive for ligation by acetone and dimethylformamide, due to a relatively large entropic effect, which is dominating when the energetic effects of ligation are small. It reflects, in quantitative terms, the control of the equatorial coordination of the Mg^{2+} ion via the axial coordination: a "hard" free Mg^{2+} ion is made into a softer center through the coordination of tetrapyrrole. Pigment structural features have comparable effects on the energetic and entropic contributions to the difference of ligation free energy between the diastereoisomers of bacteriochlorophyll. ΔS° and ΔH° values are consistently lower for the *S* epimer, most likely due to the steric crowding between bulky substituents. The two epimers show a 5 $J \cdot mol^{-1} \cdot K^{-1}$ difference in ΔS° values, regardless of the ligand type, while the difference in ΔH° amounts to 1.7 $kJ \cdot mol^{-1}$, depending on the ligand. Such steric control of ligation would relate to the partial diastereoselectivity of chlorophyll self-assembly and, in particular, the very high diastereoselectivity of the ligation of chlorophylls in photosynthetic proteins.

The coordination of ligands to Mg^{2+} ions, essential in many biological systems, can be intuitively understood in terms of interactions between hard/soft acids and hard/soft bases (HSAB model[1]). For instance, the preferential binding of water and other oxygen-containing ligands (amides, ketones, ethers, and alcohols, etc.) to Mg^{2+} is explained as the reaction of hard bases with a hard center.[2,3] This description, though only capturing qualitative characteristics, is commonly used because it seems difficult to obtain quantitative information about the coordination interactions of the Mg^{2+} ion.[2−6] These weak interactions are difficult to monitor spectroscopically because Mg is a light element with a simple electronic configuration and Mg^{2+} complexes are often kinetically too labile.[1,7]

Chlorophylls (Chls), the chief photosynthetic pigments, comprise a class of very important biological ligands of divalent Mg ion, which together form kinetically stable complexes.[8] With the incorporation of this metal center, Chls gain new coordinative properties because out of six binding positions in the first coordination sphere of Mg^{2+} ion only four are satisfied in Mg−tetrapyrrole complexes. The presence of two coordinatively unsaturated sites in axial positions determines Chl interactions with the environment (polypeptides and solvents) and thus is of great structural importance for virtually all photosynthetic Chl−proteins. This is why the axial ligation of Chls has long been a subject of extensive studies,[9−16] but surprisingly, little thermodynamic data on chlorophyll−ligand interactions can be

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found in the literature, except in the classic works of Evans and Katz[11] and Cotton et al.[12]

In the course of the chromatographic separation of plant Chls, major fractions of Chla and Chlb were always preceded by minor satellite bands (denoted as prime Chls), with spectral properties virtually identical to the main ones.[17] Only after the implementation of NMR spectroscopy were Katz and coworkers able to identify these satellite fractions as epimeric Chls, that is, as their $13^2(R)$-diastereoisomers.[18] The configuration inversion around the C-13^2 carbon, being an acid−base-catalyzed reaction, readily takes place with practically any attempt to extract Chls from their natural milieu of photosynthetic proteins.[19] Therefore, the presence of (*R*)-Chls in photosynthetic tissues was long controversial and prompted discussion as to the structure (and number of isomers) of Chls actually involved in photosynthesis, until their unequivocal detection in extracts of various photosynthetic reaction centers.[20−23] Ultimately, $13^2(R)$-Chla (prime epimer) was identified as a component of the special pair in the crystal structure of the PSI reaction center, where, with the $13^2(S)$-Chla molecule, it forms the P700 heterodimer.[24,25] Still, the finding remains somewhat puzzling, especially in light of the high stereospecificity of key enzymes involved in Chla biosynthesis/biodegradation pathways, that is, Chla-synthetase and chlorophyllase.[26,27]

The π-electron system of Chls is sensitive to changes in charge density on the centrally chelated metal.[28] In this respect, the electronic absorption features of bacteriochlorophyll a (BChla, the structure shown in Figure 1), the main Chl of photosynthetic purple bacteria,[8] provide a particularly interesting case, which can be conveniently used as a direct indicator of the central metal coordination state.[11,29,30] In the present study, spectral deconvolution of the BChla electronic absorption spectrum serves to estimate thermodynamic parameters of Chl−ligand interactions in solution and to reveal differences between the *R* and *S* diastereoisomeric forms of the pigment in coordination properties, highly relevant to the diastereotopic ligation of Chls in vivo.[31,32] On the basis of temperature effects on the spectra, we determined the ligand binding equilibria and relationships between thermodynamic parameters of ligand binding, ligand properties, and steric interactions of the side groups located near the C-13^2 center of chirality in BChla.

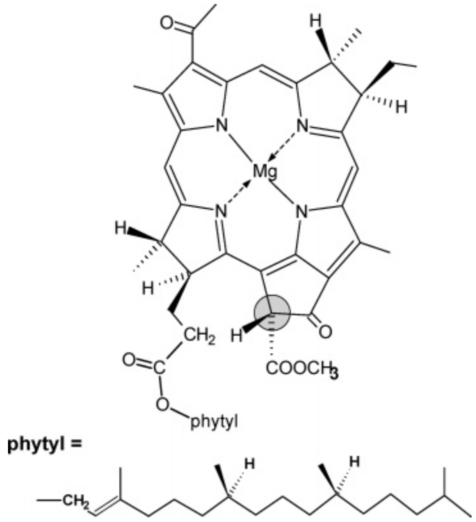

Figure 1. Structure of BChla, the main chlorophyll of purple photosynthetic bacteria; chiral carbon atom 13^2 is marked with a circle.

Materials and Methods

Pigment Preparation. BChla was isolated from wet cells of *Rhodobacter sphaeroides* following a previously described method.[33] The pigment was purified by column chromatography on DEAE-Sepharose (Sigma, Germany) and eluted with 15% methanol in acetone (v/v). A mixture of prime and nonprime diastereoisomers of BChla was obtained from the pure pigment by a 4-h treatment with a small amount of triethylamine (POCh, Poland) at room temperature. The reagent was removed in a stream of nitrogen and then under vacuum. The *R*- and *S*-diastereoisomers (epimers) of BChla were separated by HPLC on silica gel (Varian, Microsorb 100 Si column, 4.6 × 250 mm, eluent: 3% IPA in *n*-hexane, flow rate: 1 mL/min), according to a previously described method.[26] Because of a high sensitivity of the pigments to light, oxygen, and temperature, all preparatory steps were done as quickly as possible and in dim light. The pigments were stored under Ar at −30 °C.

Spectroscopic Measurements. The absorption spectra were measured at room temperature in 1-cm quartz cuvettes on a Cary 400 spectrophotometer (Varian, Palo Alto, CA). The temperature dependencies of the absorption spectra were determined on a Cary 50 spectrophotometer (Varian), in a 1-cm quartz cuvette, placed in a holder, equipped with an electronic temperature controller (Medson SC, Poland).

The ligand binding equilibria were determined in neat solvents (acetone and dimethylformamide) and in acetonitrile containing 150 mM pyridine or imidazole. The titration of BChla with aliphatic alcohols was performed at 298 K by adding the ligands (from 1.18 to 2.24 M) to the pigment solution in acetonitrile. In all experiments the same 1×10^{-5} M concentration of the pigments was used.

Solvents and Reagents. 2-Propanol, *n*-hexane, diethyl ether, and acetonitrile were of HPLC grade (LabScan, Ireland). Triethylamine, acetone, methanol, 1-propanol, and *n*-butanol were of analytical grade (POCh); ethanol, pyridine, and dimethylformamide were of spectroscopic grade (Uvasol, Merck, Germany). Acetonitrile was degassed and saturated with Ar before use. Imidazole (purity >99.5%) was obtained from Fluka, Germany.

Spectral Deconvolution. The spectra were converted into the linear energetic scale and analyzed using PeakFit software, version 4.0 (Jandel, USA). The Q_X bands were deconvoluted into a minimal number of Gaussian−Lorentzian components. Before the deconvolution, a background correction was applied using a quadratic function. The spectra recorded in acetonitrile were corrected, including into the fit also the

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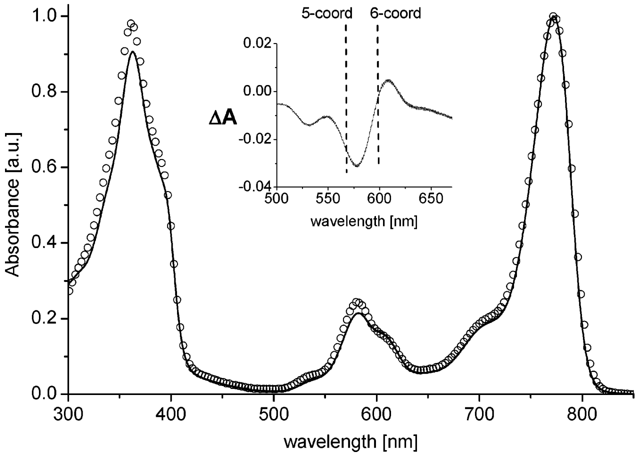

Figure 2. Absorption spectra of *R*- (—) and *S*- (○) diastereoisomers of bacteriochlorophyll a in DMF. In the inset the difference between the two spectra in the Q_X region is shown.

vibrational sidebands of the Q_Y transition. As a rule, the energies of the vibrational sidebands of the Q_X transition were placed 1000−1300 cm^{-1} higher than the origin.

Results and Discussion

During HPLC separation of the C-13^2 diastereoisomers of BChla, to our surprise, fine but meaningful differences in their absorption spectra were noted (Figure 2), which concerned mainly the Q_X absorption band, as indicated by a characteristic shape of the difference spectrum (inset in Figure 2). Because the Q_X transition in BChls is particularly sensitive to the central metal and its coordination state,[11,29,30,34] the differences possibly pointed to a nonequivalency of the diastereoisomers in interactions with axial ligands. The involvement of coordinative interactions was further confirmed by the fact that the absorption spectra of corresponding free bases (bacteriopheophytins), prepared by demetalation of the diastereoisomeric BChls, are identical (not shown). This leads to the conclusion that the relative arrangement of the C-13^2 carbomethoxyl and a bulky C_{20} moiety (phytyl) at the carbon 17^3 affects ligand coordination to BChla. A degree of steric crowding between these two moieties in 13^2(*R*)-Chla, where they are located on the same side of the macrocycle, has been detected by NMR,[35] whereas phytyl residue indeed influences interactions of Chla with solvents.[36,37]

The equilibrium between 5- and 6-coordinated forms of BChla in solution can be described as follows:

$$\text{BChla-L} + \text{L} \rightleftharpoons \text{BChla-L}_2$$

$$Q_X \approx 17\,200\ \text{cm}^{-1} \rightleftharpoons Q_X \approx 16\,400\ \text{cm}^{-1} \qquad (1)$$

where L denotes the solvent (ligand) molecule. Evans and Katz[11] and Callahan and Cotton[29] have found a direct correlation between the energy of the Q_X transition in BChla and the number of axial ligands, as indicated in eq 1. The position of the equilibrium between the two coordination forms depends on the ligand coordination strength; the binding of N-containing ligands, such as imidazole (Im) and pyridine (Py), seems strongly favored.[29] The fractions of the 5- and 6-coordinated pigment change also as a function of temperature, as shown in Figure 3. At lower temperatures, the fraction of the BChla-L_2 form grows significantly, indicating that binding of Py as the sixth ligand to BChla is exothermic. The temperature effect on the ligation is fully reversible, and the presence of a clear isosbestic point indicates that on a slow (several minutes) time scale no intermediate forms appear.

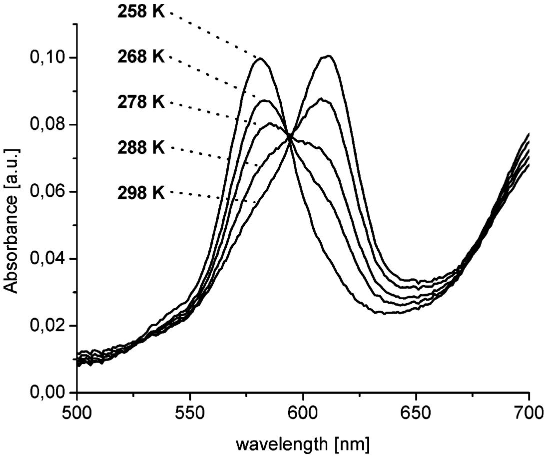

Figure 3. Shifts of the Q_X band of bacteriochlorophyll a (1×10^{-5} M) in 150 mM pyridine in acetonitrile, accompanying the temperature changes from 298 to 258 K. Other experimental details are given in the Materials and Methods section.

The equilibrium constant of ligand binding to BChla can be derived from spectral data, applying deconvolution.[11] This is demonstrated in Figure 4; the deconvolution of the Q_X band of BChla in a moderately coordinating dimethylformamide (DMF), with Gaussian−Lorentzian components as best fitting, clearly resolves two transitions comprising the band, one with λ_{max} near 16 350 cm^{-1} and the second near 17 200 cm^{-1}. Because the equilibrium position shifts toward the sixth coordinated species at lower temperatures also in this case the sixth ligand binding is exothermic.

As shown previously,[11] the areas of the resolved transitions correspond to the relative amounts between the 5- and 6-coordinated forms in equilibrium. The same approach was applied here to visualize differences in the coordination of the (*R*)- and (*S*)-BChla. The results of the deconvolution confirm that the fractions of the 5- and 6-coordinated forms in DMF are not identical for the two diastereoisomers (Figure 4). Similar differences in coordination of the BChla diastereoisomers were found in neat acetone or acetonitrile containing strongly coordinating ligands, Py or Im (not shown).

The deconvolution of BChla absorption spectra in the Q_X band region, measured over a 40 K range of temperatures (between 298 and 258 K), enabled us to determine the changes in the ligand binding equilibrium constants as a function of temperature and construct van't Hoff's plots, shown in Figure 5. In all cases, the linearity of the plots indicates that, within the applied range of temperatures, the ligation equilibrium constants are not appreciably temperature-dependent, thus facilitating a reliable determination of thermodynamic parameters of the sixth ligand binding to BChla. The thermodynamic parameters ($\Delta H°$, $\Delta S°$, and $\Delta G°$) of binding various types of sixth ligands, acetone, DMF, Im, and Py to the diastereoisomers

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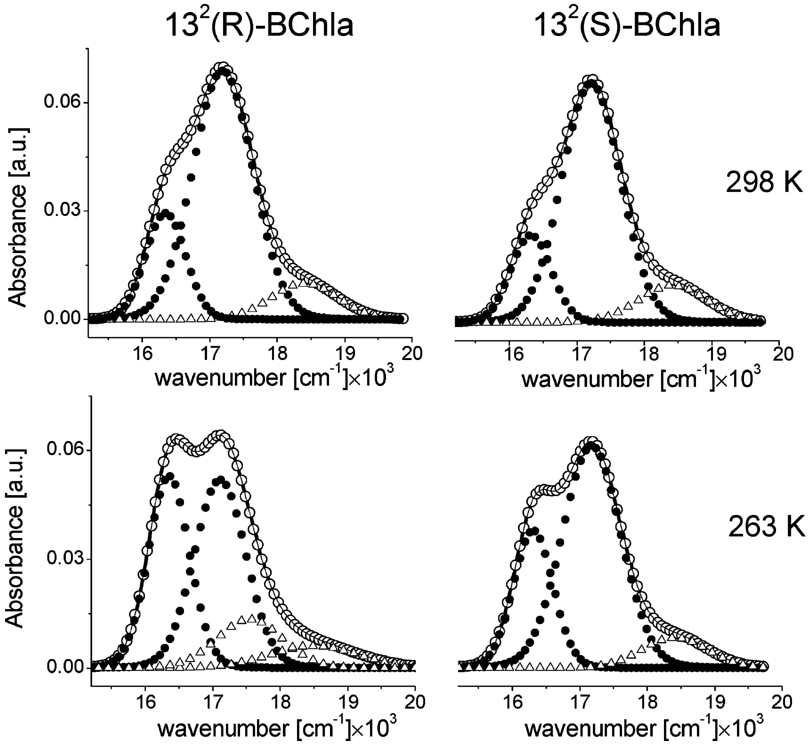

Figure 4. Deconvolution of the Q_X absorption bands of the C-13^2 *R*- and *S*-diastereoisomers of bacteriochlorophyll a in DMF at two temperatures: 298 and 263 K. The deconvolution was done with Gaussian−Lorentzian curves (see the text for details). Experimental data, ○; sum of the fitted components, —; resolved Q_X 0−0 transitions, ●; and resolved Q_X vibrational sidebands, △.

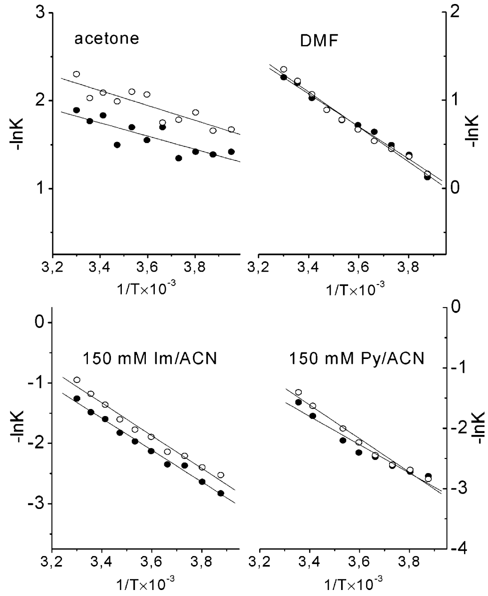

Figure 5. van't Hoff's plots for sixth ligand binding to the *R*- (●) and *S*- (○) C-13^2 diastereoisomers of bacteriochlorophyll a in neat acetone and DMF, and in 150 mM imidazole (Im) and pyridine (Py) solutions in acetonitrile (ACN).

of BChla derived from the respective plots are listed in Table 1. The Δ*H*° values are negative and strongly ligand-dependent; they decrease from −7 kJ/mol in acetone and −16 kJ/mol in DMF to as much as −23 kJ/mol for Im and Py. In acetone and DMF, Δ*S*° counterbalances a favorable (negative) Δ*H*° to result in positive Δ*G*°, rendering the ligation of BChla nonspontaneous in these two solvents. Thus, the entropic contribution becomes dominating when the energetic effects of ligand binding are relatively small. At ambient temperatures, only ligation with Im and Py occurs spontaneously as the respective Δ*G*° values reach negative values (∼−3.5 kJ/mol). Furthermore, the coordination of other oxygen-containing ligands, such as aliphatic alcohols, also seems unfavorable. Table 2 compares the changes in Δ*G*° values accompanying the attachment of Py and various alcohols as the sixth ligands, estimated from the equilibrium constants via the titration of BChla in acetonitrile. The negative value obtained for Py, which agrees very well with the one derived from the van't Hoff's plots (Table 1), shows that only the binding of Py as the sixth ligand is spontaneous at ambient temperatures. In the case of alcohols, similarly as with acetone and DMF, their detachment is spontaneous rather than their binding as the sixth ligands. Interestingly, a titration with water (not shown), even with large, 5 orders of magnitude molar excess, causes no shifts in the Q_X transition, indicating that water molecules have a very low affinity to the central Mg in BChla and can hardly be bound as the sixth ligand.

Several conclusions emerge from the quantitative data on ligand−BChla interactions, relevant to an understanding of the coordination chemistry of Mg^{2+} in biological systems, and in particular to its functioning in complexes with tetrapyrroles. First, the estimated energetic effects (Δ*H*°) of sixth ligand binding seem relatively large; for Im and Py they even approach the energies of hydrogen bonds. However, this range of Δ*H*° values corresponds to free energy penalty for removing ligands from the outer rather than from the inner coordination sphere of the Mg^{2+} ion.[3] Also, the direction of the changes in Δ*H*°, which roughly corresponds to the order of the ligand donor number,[38] is in fact opposite to that usually expected for coordination to free Mg^{2+} ion in solution[7] or in gas phase.[6] There seem to be several reasons for these changes in properties of

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Table 1. Thermodynamic Parameters of the Sixth Ligand Binding to C-13^2 Diastereoisomers (Epimers) of Bacteriochlorophyll a[a]

ligand	ΔH° (kJ/mol)		ΔS° (J/mol·K)		ΔG° (kJ/mol) at 298 K	
	R-BChla	*S*-BChla	*R*-BChla	*S*-BChla	*R*-BChla	*S*-BChla
acetone (neat)	-6.3 ± 1.3	-7.0 ± 1.2	-35.8 ± 4.9	-41.6 ± 4.3	$+10.8$	$+11.5$
DMF (neat)	-15.3 ± 0.7	-16.5 ± 0.7	-61.0 ± 2.5	-65.1 ± 2.4	$+9.3$	$+9.4$
150 mM Im in acetonitrile	-21.9 ± 0.7	-22.5 ± 1.1	-61.3 ± 2.7	-65.3 ± 3.8	-3.6	-3.0
150 mM Py in acetonitrile	-21.5 ± 1.8	-23.2 ± 1.4	-60.5 ± 6.5	-65.5 ± 5.1	-3.5	-3.7

[a] Values were derived from the van't Hoff's plots shown in Figure 5. Experimental procedures are described in the Materials and Methods section.

Table 2. Experimentally Determined ΔG_{298}° Parameters of Binding of Second Axial Ligands to *S*-BChla, in kJ/mol, Estimated via Titration of the Pigment (1×10^{-5} M) in Acetonitrile[a]

ligand					
pyridine	methanol	ethanol	propanol	*n*-butanol	2-propanol
-3.5	$+1.5$	$+2.9$	$+2.9$	$+2.8$	$+5.6$

[a] Ligand concentration: pyridine 28−150 mM, alcohols 1.176−2.24 M. Experimental procedures are described in the Materials and Methods section.

the metal center upon coordination of tetrapyrrole. One reason is that the four chelating bonds in Chls have a mixed ionic-coordinative character and the central Mg is only formally 4-coordinated in the absence of axial ligands. Further, the presently estimated ΔH° and ΔG° values obviously concern the energies of binding of the sixth ligand to the central Mg and reflect, in a way, the decrease of positive charge density on the metal due to chelation.[34] This is yet another instance of how the equatorial coordination of Mg^{2+} ion controls axial coordination: a "hard" free Mg^{2+} ion is made into a softer one via coordination of the tetrapyrrole. This observation is very much in line with the results of recent model studies on Ni-substituted BChla, which showed the influence of the equatorial ligand (tetrapyrrole π-electron system) on the partial charges at the central metal ion.[39] Because the change of the hardness of metal center is gross, the major conclusions from the present study of the sixth ligand binding can be extrapolated as well to the binding of the fifth ligand. Therefore, such modulation of the charge density (hardness) of the central Mg^{2+} in Chls seems a prerequisite for the structural functioning of these complexes in photosynthetic Chl−proteins, in which the Mg is almost exclusively 5-coordinated.[31,32] It not only widens the spectrum of acceptable coordinative bonds to amino acid residues, but also prevents saturating coordination of water molecules to the first coordination sphere of Mg^{2+} ion in Chls. In effect, it facilitates a strong coordination of Chls via nitrogen (His), oxygen (Asp, Met), and even sulfur (Met) in Chl−proteins.[24,40−43] The ease of tuning Mg coordinative properties via equatorial chelation would be then among the factors favoring the choice of that element as the metal center in Chls (with the rare exception of Zn-complexing species[44]).

A comparison of the thermodynamic parameters estimated in the present study (Table 1) shows that BChla structural features have remarkable effects on both the energetic and entropic contributions to the difference of ligation free energy between the two epimers. The ΔS° value is consistently 5 J·mol^{-1}·K^{-1} lower for the *S* form, regardless of the ligand type. ΔH° shows a similar tendency, being lower for *S*-BChla by up to 1.7 kJ·mol^{-1}, depending on the ligand. The contributions of the two parameters to the overall ΔG° difference are quite comparable as on the percentage basis the entropy changes by 7 to 16%, while the energy changes by 3 to 11%. Nevertheless, the insensitivity of the entropy part to the coordinated ligand suggests that the difference originates from the change in relative orientation of the carbomethoxyl and phytyl residues. The modifications at the periphery of the Chl molecule have been shown to strongly influence its coordinative interactions with peptide ligands.[45] Our results suggest that even relatively small structural rearrangements affect ligand binding to Chls. Since the inversion of configuration around the C-13^2 center alone is not expected to involve changes in electronegativity of the peripheral groups, lower values of ΔS° of sixth ligand binding to the *S*-diastereoisomer of BChla could be related to steric crowding between bulky substituents, which further imposes some constraints on the coordinating species.[35]

Such structural control of ligation could underlie the partial diastereoselectivity of Chl self-assembly[46,47] and, in particular, the very high diastereoselectivity of the ligation of (B)Chls in photosynthetic proteins.[31,32] Obviously, when macromolecules (polypeptides) are coordinated as ligands to Chls, the steric effects can be amplified and hence be more discriminating. This would be a reason for a highly conserved stereochemistry of Chls and a strict avoidance of an equilibrium mixture of their diastereoisomeric forms in vivo, contrary to the situation in polar solvents. A remarkable exception to this rule, with a strongly off-equilibrium diastereoisomer composition, is the P700 special pair, that is, the 13^2(*R*)-Chla-13^2(*S*)-Chla heterodimer.[24,25,31] Here, seemingly, the presence of the 13^2(*S*)-Chla is advantageous, for example, to maintain the fine structural and electronic asymmetry of the special pair, crucial to the functioning of this reaction center.[48−50]

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