

Chlorophyll *b* is involved in long-wavelength spectral properties of light-harvesting complexes LHC I and LHC II

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Received 7 February 2001; revised 5 April 2001; accepted 29 April 2001

First published online 22 May 2001

Edited by Richard Cogdell

Abstract Chlorophyll (Chl) molecules attached to plant light-harvesting complexes (LHC) differ in their spectral behavior. While most Chl *a* and Chl *b* molecules give rise to absorption bands between 645 nm and 670 nm, some special Chls absorb at wavelengths longer than 700 nm. Among the Chl *a/b*-antennae of higher plants these are found exclusively in LHC I. In order to assign this special spectral property to one chlorophyll species we reconstituted LHC of both photosystem I (Lhca4) and photosystem II (Lhcb1) with carotenoids and only Chl *a* or Chl *b* and analyzed the effect on pigment binding, absorption and fluorescence properties. In both LHCs the Chl-binding sites of the omitted Chl species were occupied by the other species resulting in a constant total number of Chls in these complexes. 77-K spectroscopic measurements demonstrated that omission of Chl *b* in refolded Lhca4 resulted in a loss of long-wavelength absorption and 730-nm fluorescence emission. In Lhcb1 with only Chl *b* long-wavelength emission was preserved. These results clearly demonstrate the involvement of Chl *b* in establishing long-wavelength properties. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Chlorophyll binding; Chlorophyll fluorescence; Light-harvesting complex; Long-wavelength chlorophyll; Reconstitution

1. Introduction

The main function of the pigments ligated to photosynthetic light-harvesting complex (LHC) consists in light capture and energy transfer within antenna complexes and in conducting the collected energy to the core components of the photosystems I and II. To fulfill these tasks the spectral properties of the pigments are influenced and tuned with respect to each other by the protein environment, resulting in a directed net energy transfer. The 10 proteins unambiguously identified as apoproteins of LHC share considerable sequence homology [1]. Conserved amino acids in the transmembrane helices are thought to serve as Chl-binding sites. These were confirmed by the structural analysis of LHC II at near atomic resolution [2]. In LHC II at least 12 chlorophylls (Chls), two luteins, one

neoxanthin and substoichiometric amounts of violaxanthin interact to achieve these functions [3]. By contrast LHC I ligates fewer Chl molecules and only one lutein molecule per apoprotein, as well as substoichiometric amounts of violaxanthin [4–6]. Despite this reduced Chl content, one subfraction of LHC I, the so-called LHC I-730, possesses special long-wavelength Chl, absorbing at around 705 nm and fluorescing at around 730 nm [7–9]. The contribution of long-wavelength Chl to total absorption appears to be species-dependent [10]. It amounts to 5% of total absorption [11] or even higher percentages in special cases [12].

The long-wavelength Chl(s) were localized to the Lhca4-subunit of LHC I-730 by different experimental approaches [5,13,14]. In a recent reconstitution analysis with truncated Lhca4 it was demonstrated that removal of 38 amino acids at the N-terminus coincided with loss of the long-wavelength fluorescence of monomeric Lhca4, which suggested that this feature was associated with Chls located at the stromal side of this LHC [15]. The function of these special Chls is still a matter of debate. It was proposed that they are involved in focusing light energy to the reaction center [16] or in photo-protection under strong illumination when the reaction centers are closed [17]. On the other hand it was also suggested that they may play a role in increasing the absorption cross-section especially in dim light, enabling organisms to live in 'shade-light environments' [12,18].

The molecular cause of this spectral peculiarity is still poorly understood. One possible explanation is the formation of a strongly coupled Chl *a*-dimer [19,20], which could result in such a pronounced absorbance shift. Koehne et al. [12], however, analyzing algae adapted to different light conditions found no indication for such a dimer formation and assumed that these special properties are due to the particular protein environment of Chl *a*. Before that, an involvement of Chl *b* in the occurrence of long-wavelength property had been inferred from time resolved fluorescence measurements by Mukerji and Sauer [21].

Regarding LHC of photosystem II (LHC II, CP29) more is already known about the relationship between Chl species and spectral properties. By variation of the supplied Chl *a/b*-ratio in reconstitution assays it was possible to reconstitute LHC II and CP29 with a constant total number of Chls but at varying Chl *a/b*-ratios [22,23]. Using deconvolution or derivative spectra it became obvious that in both LHCs a decrease in Chl *b* resulted in the loss of spectral forms absorbing at shorter wavelengths in the Q_y region. Conversely, this highlights the

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Abbreviations: Chl, chlorophyll; LHC, light-harvesting complex

importance of Chl *a* for the long-wavelength absorption in these LHCs. These findings were confirmed by analysis of reconstituted LHC II complexes lacking individual Chl molecules as a consequence of site-directed mutations [24–26].

Further insight in the relationship between individual Chl species and spectral properties can be expected from reconstitution experiments with only one form of Chl. It had been shown that Lhca4 can be reconstituted in the absence of Chl *b* [27] and Lhcb1 trimers are formed in the presence of traces of Chl *a* [23]. We therefore refolded these two proteins with exclusively one Chl species and characterized the resultant complexes with regard to pigment binding and spectral properties.

2. Materials and methods

2.1. Reconstitution of LHC

For protein overexpression the *lhca4*- and *lhcb1*-clones described earlier were used [15,28]. Total pigment extracts, individual Chls and xanthophylls were isolated from spinach or tomato leaves as in [29].

Lhcb1 was refolded from *Escherichia coli*-expressed inclusion bodies on a Ni²⁺ affinity column as described earlier [28]. Inclusion body protein equivalent to 1 mg Lhcb1 was reconstituted with 75 µg carotenoids and 0.5 mg of Chl *b* or a Chl mix with a Chl *a/b*-ratio of 1.4. The resulting complexes are designated as Lhcb1_{Chl b} or Lhcb1_{Chl a+b}. All buffers used after the folding step were kept at pH 7.5. Chl-binding protein was eluted as a green fraction from the column and loaded onto a 12 ml sucrose density gradient (5–35% sucrose, 0.06% dodecylmaltoside, 20 mM Tris-HCl, pH 7.5) and centrifuged for 20 h at 280 000×*g* and 4 °C. Lhca4 was reconstituted as described recently [15]. 167 µg inclusion body protein was used for reconstitutions with total pigment extract from tomato thylakoids equivalent to 200 µg Chl (Lhca4_{Chl a+b}) or to a mixture of individually isolated pigments. In the latter case Chl *b* was substituted by Chl *a* (Lhca4_{Chl a}) to maintain the original Chl/carotenoid stoichiometry of about 6/1. Reconstituted Lhca4 was isolated by sucrose density gradient ultracentrifugation [5]. Monomeric Lhca4 and trimeric Lhcb1 bands were collected and either used immediately or stored at –80°C.

2.2. Characterization of reconstituted LHCs

Pigment analyses of samples extracted with 80% acetone were performed by reversed phase HPLC as described in Gilmore and Yamamoto [30] and Hobe et al. [31]. Based on the well established observation that Lhca4 and Lhcb1 bind one and two lutein molecules, respectively, pigment contents are given relative to these lutein contents.

For subsequent spectroscopic analyses in the frozen state at 77 K glycerol was added (60% final concentration) to trimeric Lhcb1 or monomeric Lhca4. Absorption spectra were recorded on a UV-2101PC Spectrophotometer (Shimadzu, Japan) equipped with a home-built low temperature device. Measurements were performed with plastic half-microcuvettes (10 mm light path) at 1-nm bandwidth. Second derivative spectra were calculated by means of the Savitzky and Golay approximation which is part of the DATAMAX software (ISA Jobin Yvon-Spex, Grasbrunn, Germany). Fluorescence spectra were recorded with a Fluoromax 2 (ISA Jobin Yvon-Spex, Grasbrunn, Germany) at an optical density of the samples of about 0.1

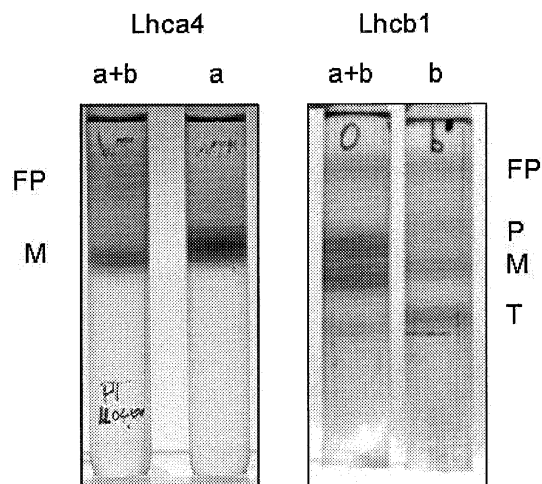


Fig. 1. Density gradient separation of Lhca4 and Lhcb1 reconstituted with carotenoids and Chl *a+b*, only Chl *a* or only Chl *b*. FP, free pigment; P, partially folded protein; M, monomer; T, trimer.

using a home-built low temperature device and polyallomer ultracentrifugation tubes. Lhca4 fluorescence emission was measured in the presence of 50% glycerol, 5 mM Tricine-NaOH (7.8) and 0.06% dodecylmaltoside. Fluorescence measurements of Lhcb1 were conducted in 60% glycerol. The bandwidth for excitation and emission was set to 2 nm.

3. Results

In order to assign spectral features of reconstituted Lhca4 and Lhcb1 to an individual Chl species, we refolded Lhca4 and Lhcb1 in the presence of carotenoids and a mixture of Chl *a* and *b* (Lhca4_{Chl a+b}, Lhcb1_{Chl a+b}), only Chl *a* (Lhca4_{Chl a}) or only Chl *b* (Lhcb1_{Chl b}). The converse experiments did not yield stable pigment–protein complexes. The reconstitution mixtures were subjected to sucrose density gradient ultracentrifugation. As is obvious from Fig. 1 omission of one Chl species resulted in the formation of pigmented bands as in the case of protein refolded with the complete set of pigments. Lhcb1 readily assembled into homotrimers, representing one state of this protein in the thylakoid membrane, whereas Lhca4 only formed monomers. In vivo Lhca4 occurs as a heterodimer together with Lhca1.

The pigment content of both complexes with one Chl species missing shows that the total carotenoid content and the number of bound Chl remained constant (Table 1). This means that the Chl *b*-binding sites in Lhca4 and the binding sites for Chl *a* in Lhcb1 are not empty, but the respective chromophores have been replaced with the other Chl species.

Table 1

Pigment composition of Lhca4 and Lhcb1 reconstituted in the presence of carotenoids and Chl *a+b*, only Chl *a* (Lhca4) or only Chl *b* (Lhcb1)

	Lhca4		Lhcb1	
	+Chl <i>a</i> +Chl <i>b</i>	+Chl <i>a</i>	+Chl <i>a</i> +Chl <i>b</i>	+Chl <i>b</i>
Neoxanthin	0.12 ± 0.02	0.05 ± 0.02	0.92 ± 0.04	0.87 ± 0.11
Violaxanthin	0.11 ± 0.02	0.17 ± 0.02	0.37 ± 0.12	0.41 ± 0.15
Lutein	1	1	2	2
Chl <i>a</i>	4.36 ± 0.32	6.11 ± 0.43	7.6 ± 0.8	0
Chl <i>b</i>	1.81 ± 0.19	0	6.1 ± 0.4	13.5 ± 0.35
Chl <i>a+b</i>	6.17 ± 0.48	6.11 ± 0.43	13.7 ± 1.1	13.5 ± 0.35

Data are given in mol/mol lutein (Lhca4) or in mol/2 mol lutein (Lhcb1) and represent means ± S.D. of four to eight experiments.

To prove the absence of Chl *a* in Lhcb1_{Chl b} samples pigment analysis was performed by HPLC in two laboratories with different protocols which both failed to detect Chl *a*. The detection limit determined by the integration of HPLC noise peaks corresponds to 0.03 molecules Chl, i.e. less than one Chl *a* molecule per 30 monomers.

For the first time, these samples allow the observation of the spectral properties of Chl *a* in Lhca4 and Chl *b* in Lhcb1 without the interference from the respective other Chl species. In Fig. 2A the 77-K absorption spectrum of Lhca4_{Chl a+b} and Lhca4_{Chl a} is shown. Monomeric Lhca4_{Chl a+b} exhibits the characteristic long-wavelength absorption extending to about 730 nm. It has the shape of a very broad peak which does not yield a visible minimum in the second derivative at the scaling used in Fig. 2A. In addition, the Lhca4_{Chl a+b} exhibits a peak in the Chl *b* region at 644 nm due to the two bound Chl *b* molecules (see Table 1) which is absent in the sample refolded without Chl *b*. The next feature is a shoulder at 661 nm evident as negative peak in the second derivative spectra, which is present in both samples. This indicates the involvement of Chl *a* in the formation of this absorption. The main absorption peak at 673 nm is shifted by 1 nm towards shorter wavelengths in the complex lacking Chl *b*. Interestingly, the shoulder at 681 nm is also weaker in this sample. At a first glance this is surprising as this is the Chl *a* region which should be fully represented in the sample folded with Chl *a*. This effect may be caused by the lack of Chl *b* and the normalization procedure which was carried out on the main absorption at 672 nm. This peak is relatively higher in Lhca4_{Chl a} due to the two additional bound Chl *a* molecules (see Table 1). The red edge of the 681-nm peak of Lhca4_{Chl a+b} is characterized by a shoulder at 688 nm as evident from the second derivative, which is more pronounced in Lhca4_{Chl a}. In summary, the main difference between Lhca4_{Chl a} and Lhca4_{Chl a+b} is the lack of the 644-nm Chl *b* peak and the absence of the long-wavelength absorption.

The absorption spectrum of trimeric Lhcb1_{Chl b} is characterized by two major peaks at 648 and 655 nm (Fig. 2B), whereas the Lhcb1_{Chl a+b} complex exhibits its typical four ab-

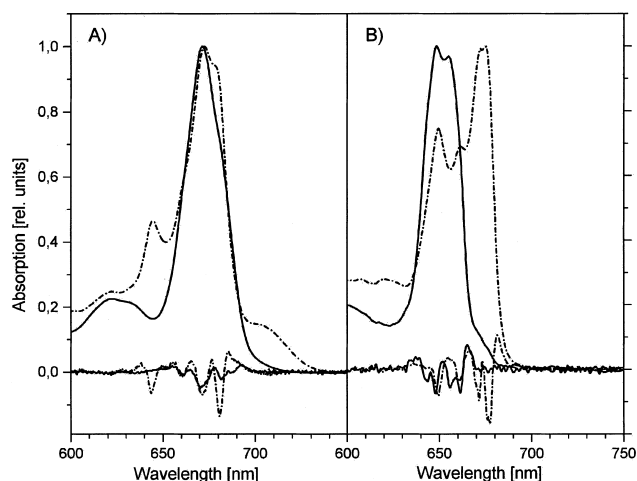


Fig. 2. Low temperature absorption spectra and second derivative spectra of reconstituted Lhca4 (A) and Lhcb1 (B). The pigment-proteins were reconstituted with carotenoids and Chl *a+b* (A+B; dashed lines) and with Chl *a* (A; solid lines) or with Chl *b* (B; solid lines). The spectra were normalized with regard to their maxima.

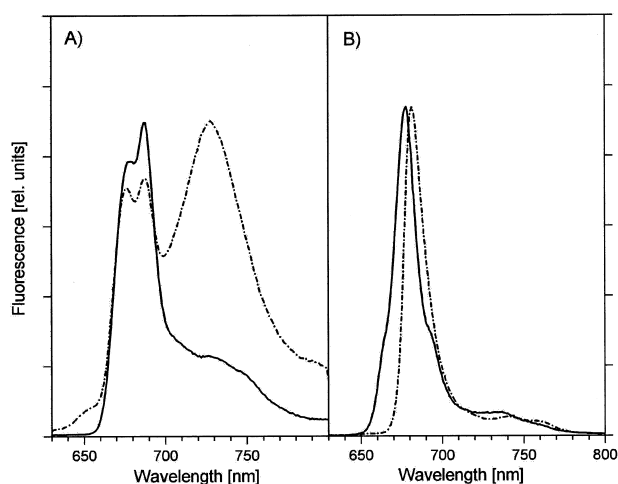


Fig. 3. 77-K fluorescence emission spectra of Lhca4- (A) and Lhcb1- (B) binding Chl *a+b* (dashed lines), Chl *a* only (Lhca4; solid line) or Chl *b* only (Lhcb1; dashed line). Excitation was at 440 nm (Lhca4) or 469 nm (Lhcb1).

sorption peaks at 650, 661, 672 and 675 nm. The second derivative analysis reveals an additional shoulder at 661 nm for Lhcb1_{Chl b}, in agreement with results from Lhcb1_{Chl a+b}. This shows that the 661-nm peak must have a Chl *b* contribution. However, a particular Chl *a* molecule was found to have its absorption band centered in this region as well [26]. In addition a shoulder at 644 nm was identified by second derivative analysis in Lhcb1_{Chl b}, which confirms the results of Nussberger et al. [32], who observed absorption bands of native LHC II in this region. On the long-wavelength edge of the spectrum of Lhcb1_{Chl b} a tail extending to about 680 nm is evident that does not yield a minimum in the second derivative. In Lhcb1_{Chl a+b} this region is dominated by the Chl *a* peaks. It should be noted that a similar feature was observed in Lhca4_{Chl a+b}, however shifted by about 50 nm towards the red edge of the spectrum.

In Fig. 3 the 77-K fluorescence emission spectra of the two LHCs are shown. Chls were excited in the Soret bands and emission was detected in the Q_y region. For Lhca4 Chl *a* was excited at 440 nm, whereas in Lhcb1 Chl *b* was excited at 469 nm. The emission spectra of Lhca4_{Chl a+b} or Lhcb1_{Chl a+b} are largely independent of the excitation being set on Chl *a* or Chl *b* (see below). It should be noted that the long-wavelength emission of Lhca4_{Chl a+b} and Lhcb1_{Chl b} is especially pronounced at low temperature.

The emission spectrum of Lhca4_{Chl a+b} at 77 K obtained after Chl *a* excitation exhibits peaks at 676, 688 and the characteristic long-wavelength emission at 729 nm (Fig. 3A). The 676-nm peak is most likely due to the emission from Chl *a* molecules that are only weakly coupled. This is the only peak which disappears upon excitation of Chl *b*. Instead a peak at 653 nm arises, characteristic for emission of uncoupled Chl *b* (data not shown). Lhca4_{Chl a} has the same principal features as Lhca4_{Chl a+b}, however the characteristic long-wavelength peak is almost absent.

In Fig. 3B a typical Lhcb1_{Chl a+b} fluorescence emission spectrum is shown with a single peak at 681 nm. Surprisingly, the spectrum of Lhcb1_{Chl b} is very similar. The fluorescence maximum is shifted only slightly to shorter wavelengths (678 nm). Uncoupled Chl *b* would in contrast yield an emission at 653

nm, which is partially evident by a broadening of the emission peak on the short-wavelength side in this sample. It exhibits also a broadening on the long-wavelength side, so that above 695 nm the spectrum closely resembles that of Lhcb1_{Chl a+b}.

4. Discussion

In the present work we demonstrate that it is feasible to reconstitute Lhca4 and Lhcb1 with carotenoids and only one type of Chl (Fig. 1). In the case of Lhcb1 it has been reported previously that the generation of stable trimers was only possible in the presence of small amounts of Chl *a* [23,33]. By employing a different protocol for refolding based on a metal affinity column we were able to generate Lhcb1 trimers at high yield with carotenoids and only Chl *b*. Such samples are required for the analysis of the spectral properties of individual Chl species in LHC.

Interestingly the LHCs produced with only one Chl species ligate the same number of Chl molecules (Table 1). Reconstitutions of Lhcb1 and Lhcb4 with both Chls at varying ratios resulted in the formation of LHC with different Chl *a/b*-ratios having bound the same total number of Chls [22,23]. These findings are explained by different binding affinities for Chl *a* or Chl *b* of individual binding sites [24,33], as it is also the case for the three carotenoid-binding sites in Lhcb1 [31]. This may lead to an uniform site occupancy in native LHC, whereas extreme, non-native pigment contents can be enforced when the complexes are folded in vitro under appropriate conditions.

Absence of Chl *b* in Lhca4 resulted in loss of long-wavelength absorption and fluorescence emission (see Figs. 2 and 3). This is in agreement with the results from time-resolved fluorescence measurements and decay-associated spectra obtained by Mukerji and Sauer [21]. They observed an increase in the amplitudes of the long-wavelength fluorescence of the two longer lived decay components upon Chl *b* excitation as compared to Chl *a* excitation. This and our results clearly indicate that Chl *b* can be involved in long-wavelength absorption/fluorescence. Chl *a*-dimers have been proposed as the origin of long-wavelength absorption and fluorescence in LHC I of spinach and maize [19,20]. In reaction center core complexes containing only Chl *a* such dimers are involved in long-wavelength absorption/emission [34]. On the basis of the data presented here we propose, however, that involvement of Chl *b* in long-wavelength emitter formation needs also to be considered in LHC I. In this context it is also interesting to note that red algae, which naturally lack Chl *b*, show only a very weak long-wavelength fluorescence [35]. Whether Chl *b* involvement in long-wavelength spectral properties is indirect by alteration of the local protein environment of the responsible Chl(s) or direct through close interaction with a neighboring Chl cannot be decided at this stage. It has been suggested that the long-wavelength band in LHC I is caused by one of the strongest coupled Chl-dimers observed in nature [20]; this could conceivably include a Chl *b* molecule. More light will be shed on this issue, when spectroscopic investigations of Lhca4 mutants devoid of individual Chl molecules are available. Disruption of the hypothetical Chl-dimer should be clearly detected, if either one of the participating Chls is lost, thereby allowing assignment of their spectroscopic and chemical nature.

Red-shifts of Chl absorption bands due to pigment–protein

and pigment–pigment interactions have been observed in the bacterial LHC, LH2 [36]. Close pigment–pigment interaction can shift the respective absorption by the coupling of excitons in the case of favorable dipole orientations. For plant LHC II early spectroscopic data were discussed in a model based on exciton coupling of Chl *b* molecules, as reviewed in [37]. With the availability of the three-dimensional structure of LHC II [2] the trimeric Chl *b* exciton model was no longer supported, but excitonic coupling between individual Chl molecules seemed highly likely. The long-wavelength Chl *b* in Lhcb1_{Chl b} may result from such an interaction. However it cannot be decided at the moment, whether this long-wavelength Chl *b* is also present in Lhcb1_{Chl a+b}. It may be present only under these particular conditions in the absence of Chl *a*. Data from mutants clearly suggest that Chl *a2* is a major component of the Lhcb1_{Chl a+b} fluorescence emitter [25,26]. Kleima et al. [23] have refolded Lhcb1 with one Chl *a* molecule per trimer. The 678-nm fluorescence of such trimers was assumed to originate from Chl *a*. This infers that the excitation energy is first equilibrated within the trimer and then emitted from a special Chl *a* molecule serving as single emitter for the trimeric complex. However, it seems more likely that each monomer of Lhcb1_{Chl a+b} possesses its own emitter: this was shown by refolded mutants lacking Chl *a2* which is present in each monomer [25,26] and has been demonstrated to be the main component of the fluorescence emitter. It is also in line with results from single molecule spectroscopy which showed the stepwise and subsequent bleaching of Lhcb1 monomers within the trimer [38]. Therefore, the 678-nm fluorescence observed in complexes containing one Chl *a* per trimer [23] may actually originate from Chl *b* and Chl *a* in those complexes.

Our results show that the absorption maxima of LHCs between 645 and 660 nm can be generally attributed to Chl *b*, whereas the absorption between 660 and 680 nm arises predominantly from Chl *a*, and therefore both Chl species contribute to absorption around 660 nm. However, Chl *b* bound in LHC can also be involved in long-wavelength absorption. This is particularly obvious for Lhca4, where the absence of Chl *b* causes the broad peak extending to about 735 nm to disappear. The existence of long-wavelength Chl *b* in Lhcb1_{Chl b} and the involvement of Chl *b* in long-wavelength properties in Lhca4 is evident in their fluorescence spectra. This is directly visible in Lhcb1_{Chl b} showing an unusual long-wavelength emission that is absent in pigment solutions. The occurrence of these Chl *b* molecules may be of particular importance for the physiological role of antenna complexes in situations where energy transfer to neighboring photosynthetic complexes is the main function.

Acknowledgements: We thank Dr. Stephan Hobe for his help with HPLC-measurements. For stimulating discussions we thank additionally Dr. Martin Lohr and Dr. Alexander Melkozernov. Financial support by the Deutsche Forschungsgemeinschaft (Schm 1203/2-1 and 2-3 and the Sonderforschungsbereich 472) is gratefully acknowledged.

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