Pigment Binding-Site and Electronic Properties in Light-Harvesting Proteins of Purple Bacteria

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Comparison of the absorption maxima with the hydrogen bonding of the C_2 acetyl groups for various mutants and wild-type bacterial antenna complexes has demonstrated a role for this molecular interaction in the tuning of the absorption properties of these complexes. There is a consistent linear relationship, between the downshift in the bacteriochlorophyll *a* C_2 acetyl stretching mode and the red shift in the absorption maximum, in both core and peripheral antenna complexes. This linear relationship allows us to estimate the contribution of H-bonding to the red shifts of these complexes and the sensitivity of the absorption to changes in the C_2 acetyl group environment. Investigation of changes in the Raman spectra caused by altering the excitation conditions reveal either no or only minor changes in the positions of the methine bridge, the C_2 acetyl, and the C_9 keto stretching modes. We interpret the lack of sensitivity of these bands to the resonance condition as indicating that neither differences in ring distortion nor in the H-bonding environment of these groups are important in determining the heterogeneous absorption bandwidth of the absorption spectrum of these complexes. We suggest that the differences in coupling between the pigments might be responsible for the static disorder and this may also be partially reflected in the small differences in the C_9 keto group stretching band we observe in the Raman spectra.

Introduction

Bacterial Antenna Structure. In purple photosynthetic bacteria, light energy is gathered by an extensive system of light-harvesting (LH) pigment−protein complexes, the spectral properties of which are precisely tuned to ensure the efficient funneling of excitation energy toward the photochemical reaction centers, where the transduction into chemical potential energy takes place. In all purple photosynthetic bacteria, the reaction center is surrounded by a "core" antenna (or LH1). In many bacteria an additional light-harvesting system exists, the "peripheral" antenna (or LH2), which transfers excitation energy to the reaction center (RC) via the core antenna. All of these antenna complexes have the same basic arrangement, being constructed from multimers of a minimal unit containing two polypeptides, α and β, to which are bound the bacteriochlorophyll *a* and carotenoid pigments. In LH1, each polypeptide binds a single BChl molecule, and these interact with each other and the protein environment to give a lower energy singlet absorption transition at ca. 880 nm. In LH2 complexes three BChl *a* molecules are bound per $\alpha\beta$ polypeptide pair.

Recently our comprehension of purple bacterial light-harvesting complexes has been greatly advanced by the publication of a number of three-dimensional crystal structures and two-dimensional projections. The LH2 complex of *Rhodopseudomonas (Rps.) acidophila* has been solved to atomic resolution,[1] and has been shown to consist of a ring of nine heterodimeric subunits. Within this annular structure the α polypeptides form an internal protein ring, while the β polypeptides form an external ring, and the bacteriochlorophyll molecules are situated between these two protein rings. A similar nonameric ring structure has been demonstrated for another LH2 complex, that of *Rhodovulum* (*Rv.*) *sulfidophilum*.[2] However in the crystal structure determined for the LH2 of *Rhodospirillum (Rsp.) molischianum*[3] the heterodimeric subunits are arranged in an eight-membered ring.

To date no atomic resolution structure for a core antenna complex has been determined; however, low-resolution projection structures have been determined from two-dimensional crystals of the LH1 from *Rsp. rubrum*.[4,5] In these structures it can be observed that the LH1 forms a larger ring than the LH2, containing 16 heterodimeric subunits. This ring is large enough to contain a reaction center which has been proposed to be located within the LH1 ring,[5] recent investigations of two-dimensional crystals of core complexes, LH1 associated with RC, from *Rsp. rubrum* and *Rps. acidophilla* confirm this location.[6,7] Though the precise three-dimensional structure of the LH1 complex is unknown, it should be possible to obtain a reasonable idea of the molecular arrangement of the binding site from the recently published crystal structure of the *Rsp. molischianum* LH2,[3] since this LH2 has both a sequence and bacteriochlorophyll binding sites that are very similar to those usually associated with LH1.[8]

Tuning of Antenna Absorption. The spectral properties of the individual pigments within the antenna complexes are strongly dependent on the interactions with their immediate environment. These interactions are responsible both for the transition frequencies of the pigments and for the static disorder which leads to an inhomogeneous broadening of the absorption transitions.

Within antenna complexes two types of specific interactions have been shown to be involved in the tuning of the absorption spectrum: first, and most apparent from the three-dimensional structure, interactions between neighboring pigments within the cyclic structure and, second, the hydrogen bonding interaction of the bacteriochlorophyll C_2 acetyl group with the protein. Coupling between pigments has long been presumed and, with the observation of van der Waals contact between the pigment molecules in the peripheral antennae, appears clear and has been shown to be important in determining the spectral properties as

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judged by a number of calculations.[9,10] Recently it has been demonstrated that a certain proportion of the red shift observed in the bacteriochlorophyll *a* Q_y transition, relative to isolated bacteriochlorophyll *a*, is due to the hydrogen bonding of the C_2 acetyl group,[11−15] and that this effect probably operates through perturbations of the electronic structure of the individual pigment molecules.[9]

Heterogeneous Bandwidth. Slow fluctuations and static disorder in the antenna complexes leads to an inhomogeneous broadening of the absorption band. Evidence for this broadening has come from a number of techniques, notably from spectral-hole burning[16−18] measurements of the homogeneous bandwidth. These measurements have allowed estimations of the inhomogeneous distribution function as a Gaussian with a width between 250 and 400 cm^{-1} for both core and peripheral antennae for their long-wavelength transition.[19−21]

This Paper. In this paper we examine the role of hydrogen bonds to the C_2 acetyl group of the bacteriochlorophyll in tuning the absorption properties of antenna pigments and examine the possible structural origin of static disorder and the heterogeneous bandwidth.

Materials and Methods

Sample Preparation. The core antenna of *Rp. rubrum* was purified from reaction center depleted chromatophores of the strain $G9^+$ as we have previously described.[22] The peripheral antenna of *Rubrivivax gelatinosus* was the kind gift of Vladimira Jirsakova and Françoise Reiss-Husson.

Spectroscopy. Raman spectra were recorded with a 90° geometry from samples maintained at 77 K in a flow cryostat cooled with liquid nitrogen (TBT, Vitry sur Seine). Ultraviolet (363.8 nm excitation, provided by a Coherent Innova 100 Ar laser) resonance Raman spectra were recorded with a Jobin Yvon U1000 spectrometer equipped with a UV coated CCD camera (Jobin Yvon Spectraview 2D). Near infrared Raman spectra, in preresonance with the Q_y transition, were recorded at 4 cm^{-1} resolution using a Bruker IFS 66 infrared spectrophotometer coupled to a Bruker FRA 106 Raman module, for these spectra excitation was provided by a Titanium-Sapphire laser (Spectra-Physics, Model 3900S) tuned to approximately 940 nm pumped with a continuous Ar^+ laser (Coherent, Innova 100). This wavelength was chosen so as to ensure preresonance with the Q_y transition and allow the collection of low-temperature spectra under similar conditions to those used to obtain room temperature spectra with 1064 nm excitation.

Results and Discussion

Linear Relation between Absorption and Down Shift. A number of experiments, that have been reported previously, have suggested that a certain proportion of the red shift in bacterial antenna complexes can be attributed to a hydrogen bonding of the C_2 acetyl group, this both in LH1[15] and LH2 complexes.[11−14] When free from interactions, this functional group gives a characteristic Raman band at 1660 cm^{-1} which is down shifted on H-bonding, in some cases by as much as 40 cm^{-1}. In the absence of a clear understanding of the physical origin of the relationship between the strength of the protein−C_2 acetyl carbonyl hydrogen bond strength and the position of the absorption maximum of the light-harvesting, we are only able to study the empirical relationship observed between the average downshift of the C_2 acetyl vibrator, a measure of the hydrogen bond strength, and the absorption maximum. However, it is unclear how this comparison is the best made.

Figure 1 displays the observed relationship between the Q_y electronic transition maximum and the average downshift of

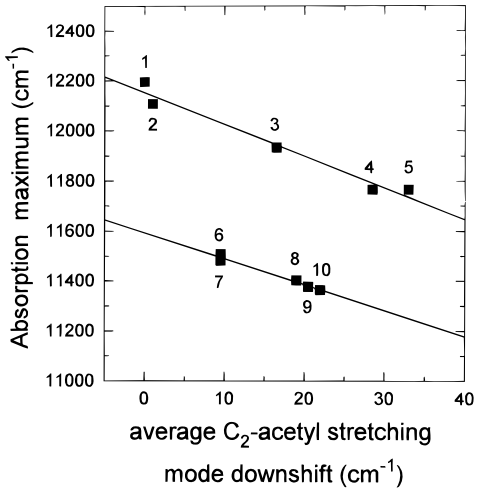

Figure 1. Relationship between the Q_y absorption maximum and the downshift of the C_2 acetyl stretching modes. We plot here the position of the room temperature near infrared absorption maximum and the total downshift observed for the two C_2 acetyl vibrators from the noninteracting frequency of 1660 cm^{-1}. The lower series of points relate to LH1 wild-type and mutants taken from Sturgis *et al.* (submitted for publication), the upper series of points show data for LH2 complexes taken from Sturgis *et al.*[12] and Fowler *et al.*[11] The lines show linear regression fits to the LH2 data and the LH1 data. The data points are as follows: 1, *Rps. cryptolactis* B800-820; 2, *Rb. sphaeroides* B800-850 mutant $F_{\alpha+13}L_{\alpha+14}$; 3, *Rb. sphaeroides* B800-850 mutant $L_{\alpha+14}$; 4, *Rb. sphaeroides* B800-850; 5, *Rv. gelatinosus* B800-850; 6, *Rb. sphaeroides* LH1 mutant $F_{\beta+9}$; 7, *Rb. sphaeroides* LH1 mutant $Y_{\beta+9}$; 8, *Rb. sphaeroides* LH1; 9, *Rb. sphaeroides* LH1 mutant $H_{\beta+9}$; 10, *Rb. sphaeroides* LH1 mutant $H_{\alpha+11}$.

the C_2 acetyl vibrator from 1660 cm^{-1}, the frequency of this vibrator when free from interaction, for a series of LH1 proteins and LH2 proteins, wild-type and mutants. The upper series of points corresponds to LH2 complexes, with points representing from right to left the 850 nm absorbing LH2 of *Rv. gelatinosus*,[13] the LH2 of *Rb. sphaeroides*, the wild-type, a mutant with a leucine at position 45 of the α polypeptide, and a double mutant with in addition a phenylalanine at position 44 of the α polypeptide,[11,23] and finally the 820 nm absorbing LH2 of *Rps. cryptolactis*.[13] The lower series of points correspond to wild-type and mutant LH1 complexes in which the tryptophans liganding the C_2 acetyl group have been changed, namely, those at position α_{43}[24] and β_{47}.[15] From this figure we have omitted three groups of data, first a number of complexes that duplicate points already included in the figure (see for example ref 13), second some mutants in which we believe there have been secondary alterations to the pigment binding site or arrangement (see ref 24), and finally the LH2 complex of *Rsp. molischianum* which appears atypical of LH2 complexes in sequence,[8] pigment binding site,[8] and structure.[3]

Both the LH1 and LH2 associated points that we show may be reasonably fitted with linear regressions, which appear as full lines on Figure 1. These two lines have similar slopes of −10.4 and −12.7, respectively. The similarity of the two slopes suggests that the effect of the protein−BChl *a* acetyl carbonyl hydrogen bond occurs through the stabilization of the lower excited electronic state of the bacteriochlorophyll monomers, relative to the ground state, and not through alterations in the degree of coupling between the different BChl *a* molecules within the protein. Indeed, if the effect were due to changes in coupling between the pigments within a ring, it might be expected to be dependent on the original degree of coupling present in complex and thus significantly larger for the core antennae than for the peripheral antennae. Thus, as we have previously proposed,[9] the strength of the hydrogen bond to the

C_2 acetyl group, as measured by the displacement of the vibrational mode attributed to this group, appears to modulate directly the absorption of the concerned BChl *a* monomer, and, via this effect, the resulting absorption of the antenna protein.

Single Mutants on Line. It is of particular interest that, for the LH2 proteins, the absorption of the mutant in which one BChl−protein hydrogen bond has been kept and one lost per $\alpha\beta$ subunit falls precisely between the LH2 complexes with none and those with two hydrogen bonds per subunit. This mutant contains alternate chromophores with and without H-bonded C_2 acetyl groups; i.e., in every possible BChl pair, the coupling occurs between molecules with different monomeric absorption properties. In a weakly coupled system, the large diagonal heterogeneity, caused by this situation, would be expected to result in a larger decrease in the red shift than that expected by interpolation between the two extremes, which both represent cases in which the absorption properties of the alternate monomers are near to identical. This is not observed, and it thus implies that the coupling between the pigments is sufficiently strong to overcome this heterogeneity between the individual chromophores; i.e., BChl *a* molecules behave at least as dimers in light-harvesting proteins.

It is instructive to compare this situation with that of the primary electron donor (P) in reaction centers of purple bacteria, which is an other red-shifted chlorophyllic structure the hydrogen-bonding patter of which has been extensively modified by site-selected mutagenesis.[25] The first results obtained on this structure were very similar with those we obtain on LH proteins; i.e., the addition of an H-bond to the P_M molecule by changing phenylalanine M 197 for a tyrosine induced a 10 nm red-shift of the Q_y electronic transition of P.[26] However, extensive mutagenesis of the aminoacids in the P binding pocket have since clearly shown that there is no clear correlation between the position of this transition and the H-bond pattern on the C_2 acetyl of this BChl dimer.[25,27] As suggested by Allen and co-workers,[28] a likely explanation for this is that the absorption of the primary electron donor of purple bacteria is dominated by the interactions between its lower energy singlet excited state and its internal charge transfer state.

No Evidence for Selection of Special Population. In view of the high sensitivity of the absorption maximum to the strength of hydrogen bonds to the C_2 acetyl groups of the bacteriochlorophylls in these proteins, we wished to evaluate the extent to which this interaction could be associated with the observed heterogeneous bandwidth of antenna complexes. To investigate this, we have compared the Raman spectra of antenna complexes obtained in preresonance with the Q_y transition and in resonance with the Soret band. While near-infrared excitation, in preresonance with the Q_y transition, will result in an increased contribution to the Raman spectra of those molecules possessing the most red-shifted Q_y transitions, Soret excitation is expected to be largely nonselective. Indeed, recent experiments performed on LH2 proteins (Gall *et al.*, unpublished results) suggest that with Soret excitation the contributions of molecules with the most blue-shifted Q_y electronic transitions might even be favored. Thus spectra obtained with 940 nm excitation should be of those molecules responsible for the redmost transitions, while spectra obtained with ultraviolet excitation should be nonselective and thus give the spectrum of a more "average" molecule. Considering the spectral inhomogeneity of these proteins as well as the relationship between their absorption maximum and the strength of the BChl *a*-protein H-bonds, a displacement of as much as 20 cm^{-1} would be expected between these two excitation conditions, if modulation of this interaction were important in determining the heterogeneous bandwidth.

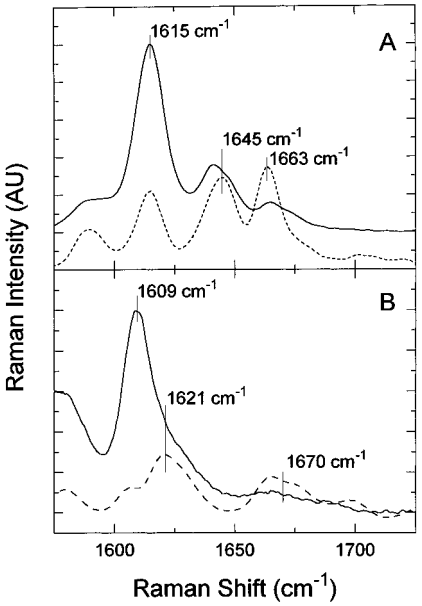

Figure 2. Low-temperature Raman spectra of light-harvesting complexes. Panel A shows spectra of isolated *Rsp. rubrum* LH1; panel B shows spectra of isolated *Rubrivivax gelatinosus* LH2. In each panel the solid line shows the spectrum obtained with 363.8 nm excitation in resonance with the Soret transitions, while the broken line shows the spectrum obtained with 940 nm excitation in preresonance with the Q_y transition.

In Figure 2 we show the spectra in the carbonyl stretching region for core and peripheral antenna complexes obtained under each of the excitation conditions. Panel A shows spectra obtained for the LH1 isolated from *Rsp. rubrum*, and panel B, from the purified LH2 of *Rv. gelatinosus*. In each case the solid line shows the spectrum obtained with UV excitation in resonance with the Soret transition and the broken line the spectrum obtained in preresonance with the Q_y transition. Clearly there are no large band displacements caused by the alteration of the excitation conditions in either complex. In the spectra of the LH1 complex four major peaks are visible in both spectra at approximately 1590, 1615 , 1645, and 1663 cm^{-1}. The three bands located at the higher frequencies have been respectively attributed to (i) the coordination sensitive methine bridge stretching mode, 1615 cm^{-1}; (ii) the C_2 acetyl stretching mode, 1645 cm^{-1}; and (iii) the C_9 keto stretching mode, 1663 cm^{-1}.[15,24,29] An additional, weak, contribution is present on the higher frequency side of the band arising from the keto carbonyl stretching mode at 1674 cm^{-1}. This shoulder is extremely weak in FT-Raman spectra (less than 10% of the integrated area corresponding to this mode), and it is slightly more evident in UV-excited Raman spectra (ca. 15% of the area of the keto carbonyl stretching band). This band has been observed in the Raman spectra of all LH1 proteins studied so far, and in an early study, it was proposed that it could arise from the keto carbonyl stretching mode of one of the two BChls present in the $\alpha\beta$ subunit.[29] However, since it is extremely weak in FT-Raman spectra, we proposed more recently that both keto carbonyl stretching frequencies are degenerate, both of them giving rise to the band at ca. 1660 cm^{-1}.[22,24] Recent studies on modified LH1 proteins seem to confirm this hypothesis.[30] We must therefore conclude that a small fraction of BChl *a* molecules are in a local environment different from that surrounding the majority of the BChl *a* molecules present in these proteins. This population has been observed both in whole membranes,[29] and in carefully isolated LH1 proteins,[22] in very similar amounts. It is thus unlikely to be associated with an artifact of purification. Precise quantification of the amount of

BChl *a* responsible for this weak signal is impossible, as the intensity of the Raman signals we observe strongly depends on the excitation conditions of the different pigments within the protein, i.e., on parameters at present completely unknown. However, this population appears minor and should not exceed a few percent of the total amount of BChl, since the shoulder is relatively weak under both excitation conditions.

As previously reported, Raman contributions of the two C_2 acetyl carbonyl groups are degenerate and contribute at ca. 1645 cm^{-1}. In the spectra of Figure 2A, however, it is clear that the band arising from these groups is composed of two constituents, of approximately equal intensity, at ca. 1640 and 1645 cm^{-1}. The presence of these two components has already been observed in the previously reported low-temperature UV-excited RR spectra of *Rsp. rubrum* LH1.[22,31] In room temperature FT-Raman spectra these components generally merge when excitation is at 1064 nm, most likely because in preresonance conditions they possess near equal intensity and thus result in a single, slightly broadened, Gaussian band; however, in our conditions of excitation, the 1645 cm^{-1} is clearly asymmetric, with a shoulder and ca. 1642 cm^{-1}. Whether this difference is due to the alteration of the excitation conditions or the reduction of the sample temperature requires further investigation.

In the spectra of the LH2 protein, peaks are visible near 1609, 1621, 1670and 1700 cm^{-1}, these have equally been assigned to the coordination sensitive methine bridge stretching mode, the C_2 acetyl stretching mode of all the BChl molecules the C_9 keto stretching mode of the molecules responsible for the 850 nm absorption, and the C_9 keto stretching mode of the molecules responsible for the 800 nm absorption, respectively.[11] As previously described,[29] in UV-excited RR spectra, the keto carbonyl contributions are extremely weak. The presence of multiple C_9 keto bands and the low intensity of these bands in UV excited RR spectra makes the observation of any possible high-frequency shoulders, as we have described above for the LH1 spectra, difficult.

It appears from comparison of the LH2 spectra that there is no detectable displacement of the peak assigned to the C_2 acetyl stretching mode toward lower frequencies on going from Soret resonance excitation to Q_y preresonance excitation. In the case of the LH1 spectra shown there appears to be a small upshift of this band, though this is attributable to slight changes in the relative intensity of its two components. However, the splitting between these two components (4 cm^{-1}) is far too small to account for the large inhomogeneous broadening of the Q_y absorption transition of this protein. According to the relationship described above, such a splitting may account for only ca. 20 cm^{-1}, i.e., less than 10% of the actually observed inhomogeneous broadening of this band. It therefore seems safe to conclude that very little, if any, of the heterogeneous bandwidth is due to energetic dispersion in the monomers due to variations in the hydrogen bond strengths to the C_2 acetyl groups. Thus, we would conclude that there is little low-frequency variation in, or conformational entropy associated with, the C_2 acetyl region of the BChl binding pockets of bacterial antenna complexes.

In Figure 2A and B, in either conditions of excitations, the position of the methine bridge stretching mode indicates in each case that the Bchl molecules are pentacoordinate.[32] The width of this band (15 cm^{-1}) as well as its stability in position according to the excitation wavelength clearly indicate that there is no heterogeneity in the Mg liganding in both of these types of proteins, i.e., that BChl−protein interactions at the level of the central ion cannot account for the heterogeneous bandwidth of their Q_y transition. It is also clear from the comparison of this peak in both cases that there is a considerable difference in its position in the two different complexes, being at 1615 cm^{-1} in the LH1 spectrum and 1609 cm^{-1} in that of LH2. In a recently submitted publication,[33] we have shown that the frequency of this mode is not directly dependent on the coordination state of the central Mg atom of the BChl *a* molecule, but rather on the size of the Bchl core, the latter being tightly dependent on the coordination state of the central ion. This situation is similar to that which has previously been observed for various porphyrins.[34] This mode may thus be used for evaluating the core size of BChl *a* molecules, with a shift of 6 cm^{-1} corresponding to a change in core size of about 0.01 Å. We may thus conclude the central magnesiums of the LH1 BChls are pulled further out of the molecular plane by the liganding histidines than those of LH2 BChls, resulting in a reduction in the core size. From previously reported spectra[22] it can be concluded that this effect is cancelled when LH1 are dissociated in the so-called B820 form and thus that it strongly depends on the integrity of the whole LH1 protein. It also is interesting to note in this regard that in a recent paper[15] we observed a splitting of this band in LH1 mutants to give two peaks at 1615 and 1608 cm^{-1} in the room temperature Raman spectra; this splitting apparently did not cause a shift in the absorption maximum.

The existence of a BChl *a* subpopulation in at least the LH1 with different keto carbonyl liganding could be associated with the inhomogeneous bandwidth of the Q_y transition. However, it has been reported that protein−BChl *a* H-bonds involving this latter chemical group had little or no influence on the absorption properties of LH complexes.[12,13,30] In particular the LH2 complexes of *Rb. sulfidophilum*[12] and *Rb. sphaeroides* (see for, e.g., ref 13) both have a hydrogen-bonded C_9 acetyl group, while the other LH2 complexes examined to date (see ref 13) have both C_9 keto groups free from interaction; these interspecific differences in the pigment environments are not associated with interspecific differences in the absorption spectra of the LH2 complexes. Therefore the weakened H-bonds to the C_9 keto group, of the population which appears to have a slightly blue-shifted transition, are probably not responsible for this spectral shift. Rather, it appears more likely that any alteration in the vicinity of the C_9 keto groups is an indirect consequence of the molecular heterogeneity responsible for the inhomogeneous bandwidth.

The molecular origin of the inhomogeneous broadening in these complexes remains to be determined. Variation in the coupling between pigments, rather than variation in the transition energy of individual pigments, appears to be the most likely source of the heterogeneity. Our failure to observe differences in the Raman spectrum that could be associated with likely mechanisms of modulation of the pigment transition energy as we changed the excitation wavelength supports this mechanism. For example differences in puckering or ruffling (which would be visible as changes in core size and hence in the position of the methine bridge stretching mode) or differences in the C_2 acetyl group H-bonding environment (which would be visible as differences in the position of this groups stretching mode) both can be excluded. On the other hand indications of differences in the C_9 keto group H-bonding environment were observed, though these are not likely to result in direct effects on the absorption.[12,22] If the molecular mechanism relies on alterations in the coupling between pigments, and hence off-diagonal disorder in the interaction matrix, it seems likely, on the basis of previous calculations,[9] that radial positional disorder in the pigments is of greater importance than either tangential positional disorder or orientational disorder.

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