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Charged amino acids as spectroscopic determinants for chlorophyll *in vivo*

(photosynthesis/electrostatic effects)

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ABSTRACT In this paper we propose that the large spectroscopic red shifts observed for chlorophyll (Chl) and bacteriochlorophyll (BChl) *in vivo* may be due to charged amino acids in the binding site. Molecular orbital calculations of the transition energies of Chl in the field of external charges are carried out. The calculated wavelength shifts induced by these charges are comparable in magnitude to those observed *in vivo*. Moreover the size of the shifts increases in the order BChl *b* > BChl *a* > Chl *a*, which is the observed trend. The ability of the calculations to account for both the absolute and relative magnitudes of the wavelength shifts argues for the validity of the model. Further indirect support comes from the recent demonstration that charged amino acids are responsible for the colors of visual pigments and bacteriorhodopsin. In addition to their effects on spectra the presence of external charges induces large changes in the ionization potential of Chl molecules and thus might explain the *in vivo* alteration of the oxidation potentials in reaction centers.

The difference between the properties of chlorophyll (Chl) molecules in solution and identical molecules *in vivo* has been a subject of great interest, extensive investigation, and much conjecture. It is clear that its local environment endows Chl *in vivo* with special physical and chemical properties, yet little is known about the nature of the interactions that produce these effects. Because it now appears that most if not all of the Chl in photosynthetic systems is intimately associated with proteins (1), it seems appropriate to consider how specific amino acids might affect the behavior of bound Chl. Properties that are strongly influenced by the protein environment include redox potentials, photochemically induced electron transfer, and absorption maxima. Of these, the latter are easiest to characterize and interpret because spectroscopic transition energies for conjugated systems may be calculated theoretically with considerable accuracy.

Chl *in vivo* and in isolated lipoprotein complexes absorbs light at longer wavelengths than does monomeric Chl in simple organic solvents (2). The magnitude of the shift varies considerably among species and somewhat less for different pigments within a single organism (comparing, for example, reaction center with antenna Chl). In green plants the magnitude of the wavelength shift is between 300 and 800 cm^{-1}. In the case of photosynthetic bacteria the shifts are often much larger, reaching 1,500 cm^{-1} for bacteriochlorophyll (BChl) *a*-containing species and as much as 2,700 cm^{-1} for the antenna complex of the BChl *b*-containing species *Rhodopseudomonas (Rps.) viridis* (2). Although the origin of these shifts is a question of some interest in its own right, its more general relevance owes to the possibility that the shift is due to highly specific and functionally important Chl–protein interactions. Thus, identifying the source of the large *in vivo* red shifts may be an important step in elucidating the mechanism of electron transfer and energy utilization in photosynthetic systems.

Several models have been proposed to explain the red shifts but it has been generally recognized that these are unable to account for the full magnitude of the effect. For example, the shifts appear too large to be explained in terms of bulk "solvent" effects (3), where the protein is viewed simply as an alternate solvent for the Chl. A more frequently cited proposal involves exciton interactions among two or more Chls. However, there is good evidence that, at least in some cases, Chl–Chl interactions account for only a part of the observed red shifts (4). First, in the BChl *a*–protein complex from *Prosthecochloris aestuarii*, whose structure has been determined (5), the Chl molecules are too far apart to account for more than 150 cm^{-1} of the observed 640-cm^{-1} shift (5, 6). Second, in partially oxidized chromatophores from cartenoidless mutant strain R26 of *Rps. sphaeroides* it appears that *monomeric* BChl absorbs at 852 nm, shifted by over 1,230 cm^{-1} from its absorption maximum in organic solvents (7). Finally, as has been emphasized by Pearlstein (see also below), it is difficult to account for reaction center spectra solely in terms of exciton interactions.

One mechanism for the *in vivo* red shifts that has only recently been considered involves electrostatic interactions between bound Chl and charged amino acids on the protein (8, 9). A rationale for this suggestion is the recent demonstration that charged amino acids are responsible for the 2,700-cm^{-1} red shift involved in the formation of bovine rhodopsin (10) and the 5,000-cm^{-1} red shift involved in the formation of bacteriorhodopsin (11). Because electrostatic interactions also appear to play a central role in the photochemical energy storage mechanism of retinal based pigments (12) it seems reasonable to consider their effects on the physical and chemical properties of protein-bound Chl.

METHOD OF CALCULATION

The spectral transition energies were calculated with the CNDO/S method of Del Bene and Jaffe (13–15), with semiempirical parameters taken from the work of Ellis *et al.* (16), and the repulsion integrals were calculated with the Mataga approximation. Charged amino acids were represented, as in our previous work, as external point charges. These are likely to be present as members of an ion pair in a "salt-bridge." The interaction of charges with the molecular electronic distribution was included in the diagonal elements of the Fock matrix as discussed previously (17). The 130–150 lowest energy configurations singly excited with respect to the self-consistent field ground state were included in the configuration interaction expansion. This proved to be sufficient to assure convergence. This method has previously been applied with limited success to metalated porphyrin spectra (18, 19). Our calculations on such

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Abbreviations: Chl, chlorophyll; BChl, bacteriochlorophyll; *Rps.*, *Rhodopseudomonas*.

compounds, which will be presented in a separate publication, demonstrate that reasonable predictions of Mg and Zn porphyrins can be obtained if the compound is modeled as a dianion interacting with a central +2 point charge.

The geometry used for each molecule in these calculations was based on the x-ray structure of Fischer *et al.* (20) for methyl pheophorbide *a*. The bond angles and bond lengths reported by that group are used, but all torsional angles are set to either 0° or 180°, except for hydrogen atoms on saturated carbons, forcing the entire macrocycle to be planar. The hydrogens on saturated carbons are given a tetrahedral H-C-H angle and are symmetrically out of plane. All C—H bond lengths are set to 1.08 Å, and the hydrogens are assumed to bisect the C-C-C angle containing them. The geometries of BChl *a* and BChl *b* are taken directly from that used for Chl *a* and are identical to it in all comparable positions. The C═O bond length is taken to be the same as the C═C length in the vinyl group of Chl *a*. The extra C═CH on BChl *b* has a bond length of 1.2 Å. This group is assumed to be coplanar with the rest of the macrocycle. These structures are shown in Fig. 1. In what follows, the terms Chl *a*, BChl *a*, and BChl *b* shall be interchangeably used to identify either the naturally occurring pigments or the moieties used in this study, the distinction being obvious depending on whether the calculated results or the experimental data are being discussed.

RESULTS

In a recent study Davis *et al.* (9) reported the synthesis of a 3a-amino derivative of Chl *a*, which proved to have an absorption spectrum very similar to that of the native Chl *a*. The amino group can be protonated at appropriate pH, thus providing a reversibly placeable charge near the macrocycle periphery. [Similar experiments were used to detect through space electrostatic interactions in visual pigment analogs (21).] Because the nitrogen is one carbon removed from the conjugated ring system it should have little effect on the spectrum, as is borne out by the absorption spectrum in basic solution. Any change in the spectrum in acid solution should be due to the charge of the protonated amine. In fact, the absorption maxima reported, extrapolated to complete protonation and complete deprotonation, indicate a 115-cm^{-1} blue shift for the charged species. This is somewhat greater than the 90-cm^{-1} blue shift quoted by the authors, which was based upon the maxima at the extremes of pH actually used, rather than the extrapolated value (9).

These measurements provide a means of testing the calculated results on a well-characterized system. The protonated amino group was represented with a +1 point charge centered on the position appropriate for the nitrogen atom by using C—C and C—N bond lengths of 1.5 Å and a tetrahedral C-C-N angle for the 3a-$CH_2NH_2^+$ group. The difference between the calculated transition energy and that obtained for the isolated Chl *a* molecule (i.e., without the charge) corresponds to the spectral shift. There remains one undetermined geometric parameter in the above description, that being the torsional angle between the C-C-N plane and the plane of the macrocycle. A value of $\gamma = 0$ is taken to correspond to a *cis*-conformation of the C(1)-C(3a)-N(3b) chain. The shifts have been calculated for $\gamma = 0°$, 90°, and 180°, giving blue-shifted transitions by 336 cm^{-1}, 244 cm^{-1}, and 127 cm^{-1}, respectively. These are in reasonable agreement with the experimental results that correspond to an average over the entire range of γs. It should be pointed out that the protonated species that is detected in these experiments is associated with a negative counter ion. This will reduce the effect of the positive charge. In a protein environment, external charges in the form of ionic amino acid groups could have their counter ions constrained to be further away than counter ions are in solution, where they are free to move.

Although the agreement with the experimental results (9) is encouraging, the particular charge placement was dictated by ease of synthesis and thus does not reveal the range of magnitudes that can be expected for charge-induced spectral shifts. In fact, this position produced one of the smallest shifts we calculated. The difficulty in synthesizing compounds with various charge placements makes a computational examination of the possibilities desirable. The distance of the charges on the protein from the Chl molecule will be greater than in the case of the Chl *a*-amino analogue, where the 2- to 2.5-Å distance is the result of covalent linkage of the amino group to the macrocycle (9). In a protein–chromophore complex the distance will be characteristic of van der Waals interactions, and a range of 3–3.5 Å is more reasonable. With this in mind, the distance from the point charge to the closest point on the molecular plane has been set in all cases to 3.5 Å. Various positions at 3.5 Å out of the plane of the macrocycle have been chosen in each of the three molecules in order to identify the locations most susceptible to external charges.

The search for the largest spectral shifts, though not exhaustive, did produce a number of striking results. The spectral shifts as a function of charge location for Chl *a*, BChl *a*, and BChl *b* are presented in Table 1. Although the calculated values

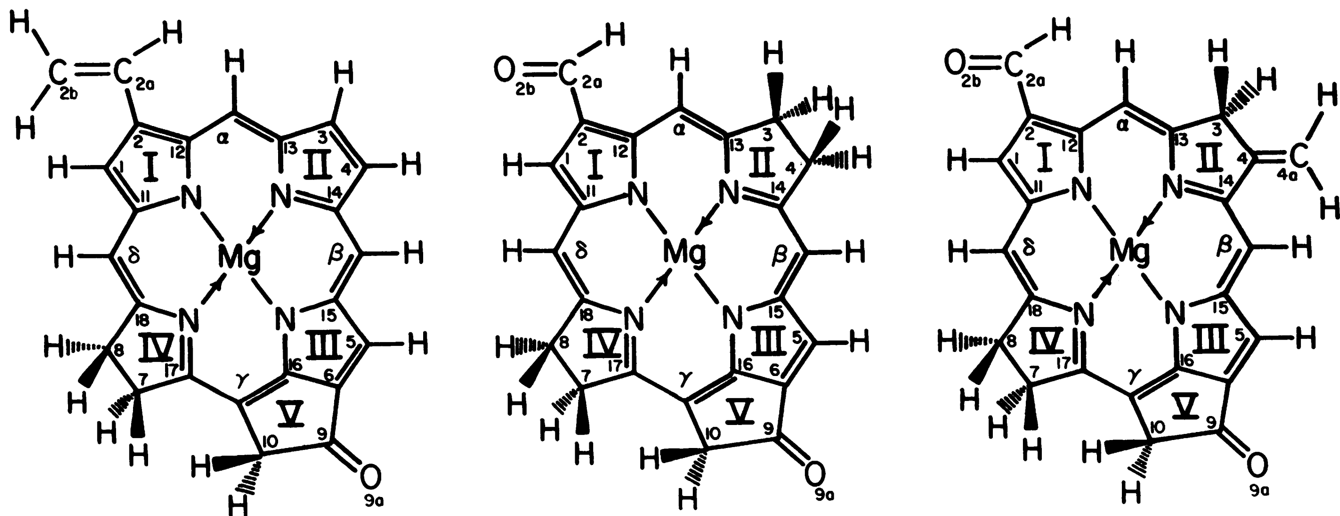

FIG. 1. Structure and numbering systems for the Chl *a* (*Left*), BChl *a* (*Center*), and BChl *b* (*Right*) moieties used in the calculations.

of λ_{max} appearing in Table 1 do not exactly match the experimental ones, the agreement is reasonably good and is considerably better than those produced by previous *ab initio* and semiempirical calculations. We have made no attempt to reparameterize the method to reproduce the specific absorption maxima of Chls and have used the values used in previous CNDO/S studies (16). The present results are considerably better than previous CNDO/S (18, 19) and PPP (22) calculations of Chls, porphyrins, and so on because of the incorporation of the effects of the central metal atom. They are also a great improvement over *ab initio* calculations of the spectral transition energies (9) due to the inability of such methods to produce a balanced treatment of ground and excited states at the present level of calculational technology. The smaller discrepancies between the results presented here and the experimental values are not worrisome because we are basing our calculations on slightly modified chromophores and because we are interested in the *shifts* produced by external charges rather than the actual values of λ_{max}. The calculated magnitude of the shifts should not be affected by small changes in the calculated λ_{max} of the isolated molecule—an expectation that has been repeatedly substantiated in previous work on visual pigments. A final point that should be made concerns the relative absorption maxima of the isolated molecules. The red shift in going from Chl to BChl *a* is reproduced in our calculations, but we calculated a blue shift in going from BChl *a* to BChl *b*. We do not consider this to be a critical failing in the procedure, because the energies involved amount to <500 cm^{-1}. Such an error could easily be the result of inaccuracies in geometry around the exocyclic double bond, which is not well known and which can be expected to produce a red shift of $\approx$1,000 cm^{-1} due to strain induced in the adjacent ring (23). This effect, if accounted for in our calculations, should lead to a value of λ_{max} for BChl *b* about 30 nm to the red of that for BChl *a*, in agreement with the experiment. The largest shifts found in our calculations were $-1{,}346$ cm^{-1} for Chl *a*, $-1{,}404$ cm^{-1} for BChl *a*, and $-2{,}074$ cm^{-1} for BChl *b*. In the case of Chl *a*, the calculated shifts are more than sufficient to include the entire range of *in vivo* shifts normally found. For BChl *a* and BChl *b* the calculated shifts are somewhat smaller than the largest *in vivo* shifts of $-1{,}500$ cm^{-1} and $-2{,}700$ cm^{-1}, respectively. We do not feel that this discrepancy is significant because larger shifts can be produced by bringing the charge a little closer than 3.5 Å or by the reasonable assumption of a distance-dependent dielectric constant within the protein (10). Moreover, there are enough uncertainties inherent in the calculations that differences of a few hundred cm^{-1} should not be viewed as significant.

Table 1. Absorption maxima and spectral shifts for Chl *a*, BChl *a*, and BChl *b* in the field of external point charges

	Chl *a*		BChl *a*		BChl *b*	
Geometry*	λ_{max}, nm	$\Delta\nu$, cm^{-1}†	λ_{max}, nm	$\Delta\nu$, cm^{-1}†	λ_{max}, nm	$\Delta\nu$, cm^{-1}†
No charge‡	605	—	742	—	717	—
−1 (O-9a)	625	−532	796	−915	773	−1,004
−1 (C-15)	623	−482	808	−1,107	788	−1,250
−1 (C-6)	626	−577	806	−1,076	783	−1,171
+1 (C-1)	633	−740	828	−1,393	818	−1,726
−1 (C-5)	625	−535	812	−1,154	792	−1,311
+1 (C-1), −1 (C-5)	658	−1,346	828	−1,404	833	−1,950
−1 (C-4a)					783	−1,172
−1 (C-4a), −1 (C-5)					842	−2,074

* The geometry is specified by the nearest atom (in parenthesis) to the charge (sign and magnitude given before parenthesis). The charge is positioned 3.5 Å out of the molecular plane above the listed atom.
† A negative $\Delta\nu$ corresponds to a red shift.
‡ Reference for spectral shifts.

The largest shift shown in Table 1 is for BChl *b*, as is true *in vivo*. It is produced by two negative charges, one above C-4a and one above C-5. C-4a is a position that is not conjugated to the macrocycle in either Chl *a* or BChl *a*. For these molecules the most effective geometry corresponds to a +1 charge above C-1 and a −1 charge above C-5. The sensitivity of the spectral shifts to external charges placed near C-4a would tend to suggest that strong electrostatic interaction located near this position is responsible for the large wavelength shifts observed for photosynthetic bacteria containing BChl *b*. In fact, the situation is much more complicated as is evident from this observation. The entire macrocycle of BChl *b* is rendered more sensitive in a spectroscopic sense by the additional exocyclic double bond. Similarly, the macrocycle of BChl *a* is more sensitive to external charges than that of Chl *a*.

The red shifts in the absorption maxima are not the only manifestations of the protein environment of the chromophore. Another important alteration of Chl properties is a change in the oxidation potential. For instance, the P_{700} electron donor complex of the green plant photosystem I has an oxidation potential about 0.420 V less than that of Chl *a in vitro*, whereas the corresponding photosystem II pigment P_{680} is 0.1–0.2 V harder to oxidize than solvated BChl *a*. Koopman's theorem defines an approximate ionization potential for a molecule as the eigenvalue of the Fock matrix corresponding to the highest occupied molecular orbital. We have found the effect of an isolated external charge 3.5 Å from the chromophore to be an $\approx$2-eV decrease in the ionization potential for a negative charge and an approximately equal increase for a positive charge. These values are not meant to be predictions of changes in oxidation potentials, but simply a demonstration that, as would be expected, external point charges will affect these quantities.

DISCUSSION

The results presented in Table 1 demonstrate that the magnitude of the red shifts observed for Chl *in vivo* can be accounted for by electrostatic interactions between the Chl and nearby charged amino acids on the protein. Electrostatic Chl–protein interactions are also successful in accounting for the relative spectral shifts observed for different types of Chl. Taken together these results suggest the existence of charged amino acids in the binding sites of at least some Chl-containing proteins. We cannot, based on available evidence, identify the specific interaction site. However, our results do implicate certain positions along the macrocycle as particularly sensitive ones to external perturbations.

As discussed above, the red shifts observed for Chls *in vivo* have frequently been attributed to "coupled oscillator" interactions. Although these may play a crucial role in some reaction centers, the large (12 Å) separation of BChl *a*s in the antenna complex of *P. aestuarii* (5), and presumably in other antenna complexes, suggests that Chl–Chl interactions play only a secondary spectroscopic role. In these systems protein-induced spectral shifts are clearly indicated. An experimental test of this hypothesis will be available when the high-resolution crystal structure of this protein is solved. For this system the observed red shift is 600 cm^{-1}, which is considerably smaller than the largest shifts we calculate for BChl *a*, and thus we would predict the presence of one or more charged or perhaps polar amino acids in the Chl binding site. A calculation of the BChl *a* transition energy in the specific environment of that protein would clearly be of considerable interest. However, this work must await a sequence determination.

A second prediction of our calculations is that if different Chls could be bound to an identical protein, the red shifts vary

according to the order BChl b > BChl a > Chl a, assuming that all three molecules occupy nearly equivalent positions in the binding site. This is not meant to imply a common protein environment for all Chls, but rather, for example, that if the chromophore of a BChl a-containing complex were replaced with Chl a, the spectral shift would be decreased. Recently, Clayton and Clayton (24) demonstrated the reversible dissociation of a BChl a-protein complex. The results show the feasibility of competitive binding experiments with a variety of Chl analogs, such as have been used with rhodopsin (10, 11). These could provide important tests of our model. Further studies on Chl-containing proteins together with efforts to study the interaction of Chl with different proteins (25, 26) and to simulate Chl–protein interactions (9) should considerably extend our understanding of environmental effects on Chl spectra.

The possible role of protein-induced spectral shifts in photosynthetic reaction centers is difficult to assess, partially because of the existence of large Chl–Chl interactions. There is wide support for the hypothesis that two of the four BChls in bacterial reaction centers form a "special pair" that corresponds to the primary electron donor. The strongest evidence comes from ESR and electron nuclear double resonance studies on the radical cation that forms in the reaction center of *Rps. sphaeroides*. The ESR lines narrow (27) by $1/\sqrt{2}$ and the separation between electron nuclear double resonance lines decreases (28, 29). Both of these effects are expected if the unpaired electron is delocalized over two BChls, hence the special pair. The situation for green plant reaction centers is somewhat different. Recent ESR data argue against a dimer model in this case (30). Moreover, it has been recently demonstrated that the magnetic properties of P_{700} can be closely mimicked by an enolized tautomer of Chl *a in vitro* (31), eliminating the need to invoke a dimer model. Even for bacterial reaction centers the special pair hypothesis is not without its difficulties, as has been emphasized by Pearlstein (32). [See also the recent review by Parson (33) for an extended discussion.] One underlying problem is that it has not been possible to provide a consistent explanation of reaction center spectra both before and after the oxidation of the *Rps. sphaeroides* P_{870} and *Rps. viridis* P_{960} primary electron donors.

Limiting discussion to the primary donors, their long-wavelength absorption maxima could in principle be due to coupled oscillator interactions, spectral perturbations by charged amino acids, or some combination of these effects. However, it is worth noting that the spectral shift of BChl b in P_{960} is significantly larger than that of BChl a in P_{870}. This could be due to stronger Chl–Chl interactions in this case, but the absence of magnetic resonance evidence for dimer formation in *Rsp. viridis* suggests, if anything, weaker coupling between chromophores in that system. Electrostatic interactions would thus appear to be implicated.

Some support for the existence of a charged group near P_{870} comes from the recent measurement by Feher and co-workers of electrochromic band shifts in reaction centers (34). The shift for P_{870} is considerably larger than that for other reaction center Chls, which is consistent with the polarizing effects of an electrochromic charge. A related role for charged amino acids in the electrochromic response to cartenoids *in vivo* has recently been suggested (35).

Finally, it is clear that the presence of charged groups in reaction centers can affect electron transfer reactions in a number of ways. See, for example, a recent conjecture by Warshel in this regard (8). In fact, our calculations suggest that the changes in ionization potentials are greater, in energetic terms, than the spectroscopic shifts. Although, as discussed earlier, the oxidation potentials have not been calculated, it is reasonable to expect that the proximity of charged residues to the chromophore could greatly affect both the potentials and the kinetics of electron transfer. The possible role of charged amino acids in the primary process in photosynthesis appears then to be a question of some relevance.

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