fect the free energy of one or both of the hs heme derivatives involved. Other interpretations, however, might give a quite different picture of the influence of the T–R switch. For example, the affinity decrease in T can also be described by assuming a spin-state change and a modest decrease in IHP affinity upon conversion to T. In order to complete the description of the free-energy balance in carp Hb, a complete study of N_3^- and IHP binding to carp met-Hb would be required, including the difficult task of looking for cooperativity in N_3^- binding and/or studying the Hb/met-Hb(N_3) redox equilibrium.[45]

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Possible Interpretation of Long-Wavelength Spectral Shifts in Phytochrome P_r and P_{fr} Forms

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Abstract: Semiempirical calculations of the chiroptic properties qualitatively support the anionic form of phytochrome P_{fr} proposed by Rüdiger and co-workers, in which conjugation between rings A and B is interrupted. Among the infinity of conceivable skewed conformations of the chromophores of P_r and P_{fr} some are found which fit the observed chiroptic data in a consistent fashion. It is concluded that the loss of conjugation between rings A and B entails a change of conformation on going from native P_r to native P_{fr}.

I. Introduction

A remarkable fact in nature is the analogy between photochemically active pigments in plants and oxygen-binding pigments and their breakdown products in animals. We have previously[1,2] studied the long-wavelength chiroptic properties of gall pigments specifically bound to serum albumin. From a semiempirical quantum-chemical interpretation of the results we have drawn conclusions on possible conformations of bilirubin and biliverdin in their protein complexes, hoping to contribute to the understanding of the transport and transfer mechanisms of these molecules in vivo. In the present investigation we consider photochemically active phytochrome,[3] the structure of which is closely related to biliverdin and which, in the plant cell, is also specifically bound to a protein. Two

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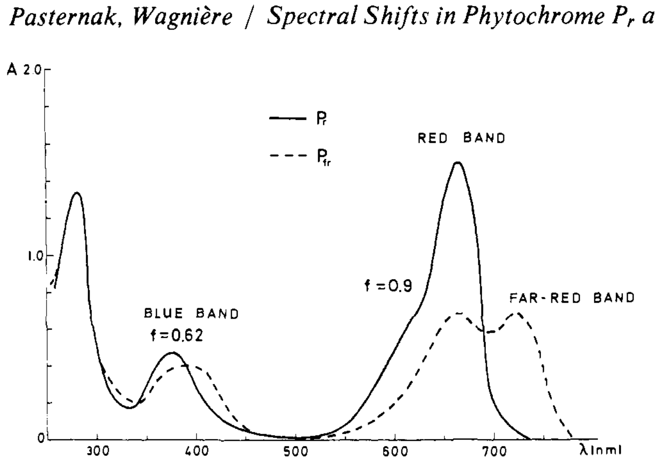

Figure 1. Experimental UV-vis spectra of P_r (—) and P_{fr} (- - -), redrawn from ref 3.

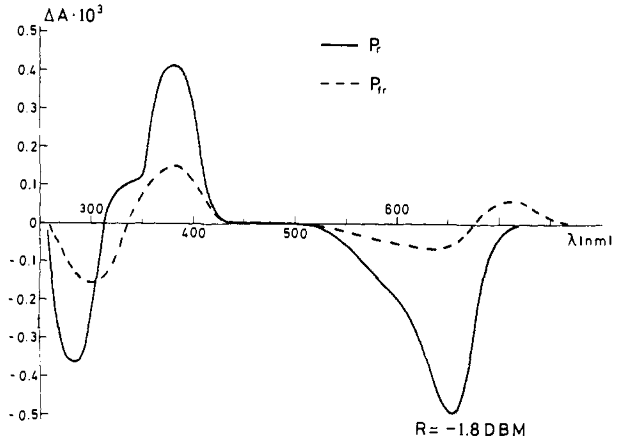

Figure 2. Experimental CD spectra of P_r (—) and P_{fr} (- - -) redrawn from ref 3.

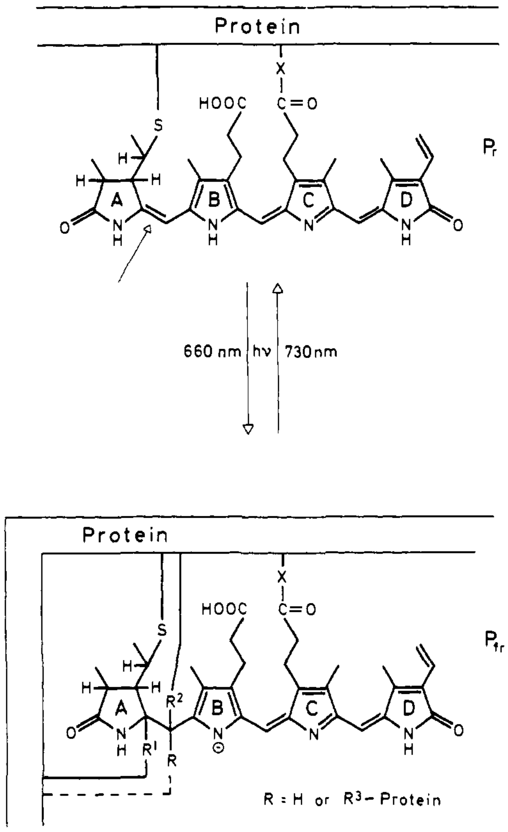

Figure 3. Proposed[13,22] structures of P_r and P_{fr}. R^1 and R^2 represent protein functional groups. R stands for a possible third protein functional group R^3 or, alternatively, for H.

distinct forms of phytochrome are spectroscopically well characterized: P_r, the "red" form, and P_{fr}, the "far-red" form.[3-8] Native P_r converts to P_{fr} under irradiation at $\lambda \simeq 660$ nm. Native P_{fr} reverts to P_r under irradiation at $\lambda \simeq 730$ nm, as well as thermally, implying that native P_r is the more stable form thermodynamically. The conversion of native P_r to P_{fr} does not seem to be elementary, but to proceed through alternate pathways and several short-lived intermediates, which can be detected at low temperatures.[5-8] Different possibilities have been proposed to account for the interconversion, and they can be classified broadly in two main schemes: (1) isomerization of the chromophore[5] through H-atom migration or intramolecular proton transfer;[9,10] (2) a change in the mode of binding to the protein, involving either an intramolecular charge transfer state[11] or an intermolecular proton transfer, leading to an ionized chromophore[10-13] stabilized by the apoprotein. Furthermore, it is suggested that some conformational change occurs in the protein upon photoconversion, but the evidence in this respect is contradictory.[8]

Recently, Rüdiger and co-workers[13-18] have proposed structures for the P_r and the P_{fr} chromophores, which have found indirect support in the work of Scheer and co-workers.[19-22] Assuming the proposed P_{fr} structure to be correct, we wish here to discuss the interpretation of the long-wavelength spectral shifts between P_r and P_{fr} and, in particular, the possibility that the chromophore of native P_{fr} is stabilized as an anion. Furthermore, we suggest possible conformations of these chromophores in their native, protein-bound states.

II. Spectroscopy and Chemistry of Phytochrome

UV-vis and CD spectra of P_r and P_{fr} have been reported in the range of 250–800 nm.[3,6] These spectra, typically resembling those of other bile pigments like biliverdin, are shown in Figures 1 and 2. Native P_r presents a strong, broad absorption at 660 nm ($f_1 \sim 0.9$;[6] the "red" band) with a shoulder at 620 nm. There is a peak at 380 nm ($f_2 \sim 0.6$;[6] the "blue" band) followed by a stronger, sharper absorption at 270 nm. The oscillator strength ratio f_2 (380)/f_1 (660) is reported to be ~0.7 in ref 6, but is apparently less according to ref 3 (Figure 1) and is sensitive to the conditions of measurement. The Cotton effect of the red band is negative ($R_1 \simeq -1.8$ DBM[6]); that of the blue band, where two peaks seem to be resolved in CD, although only one appears in UV, is positive.[3] No numerical value for the rotatory strength R_2 of the latter band is reported in the literature, but the ratio $|R_2(380)|:|R_1(660)|$ can be estimated as 1.2. The 270-nm band presents a negative Cotton effect.[3] Conversion to native P_{fr} causes the appearance of an additional peak at 720 nm (the "far-red" band), the rest of the spectrum being only slightly affected. The Cotton effect of the "far-red" band is positive[3] (estimated value $R_1' = +0.2$ DBM, based on R_1 (P_r) = −1.8 DBM). While it is beyond doubt that these long-wavelength transitions originate in the pigment chromophore and are of pseudo-π-π^* type,[19,23] the question remains as to whether the 270-nm band may not be due to the protein.[3]

The effects of denaturation on the biliprotein spectrum have been studied[6,13] and it is possible to conclude that the chromophore conformation is more "open" in native P_r than in the denaturated compound.[1,2,6,22] The structure suggested by Rüdiger and co-workers[13] for the P_r chromophore, in particular the hydrogenated ring A (see Figure 3), has recently found further direct support.[14-18]

Denaturation studies on P_{fr}[13] and the evidence concerning the structure of P_r seem to indicate that a double bond has disappeared (see arrow in Figure 3) upon conversion of P_r to P_{fr}. The chromophore would thereby be divided into two in-

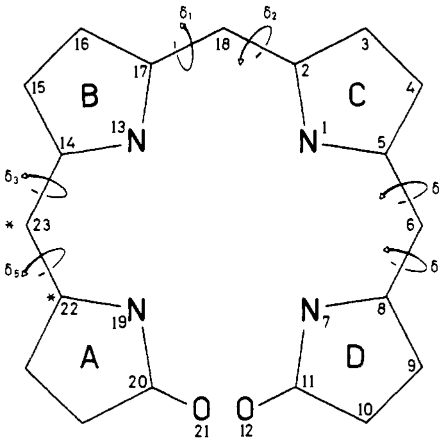

Figure 4. Numbering of atoms and definition of dihedral angles δ_i in the P_r and P_{fr} model chromophores. Starred atoms 22, 23 do not contribute to the (pseudo) π system in the proposed[13,22] P_{fr} structure and are not considered in the PPP-type model calculations on P_{fr}.

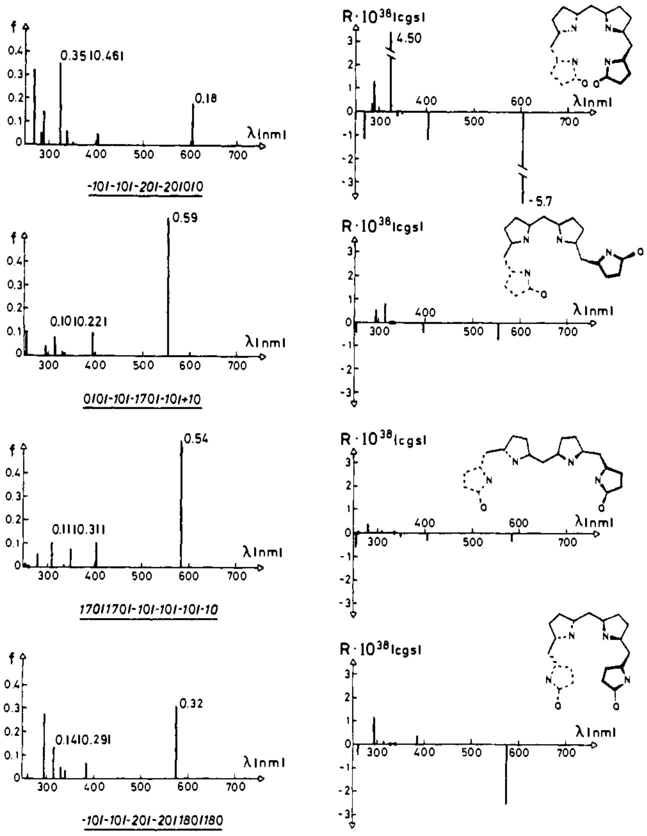

Figure 5. Computed UV-vis and CD spectra of P_r in four representative conformations. From top to bottom: (1) "closed" or "ring-like", (2) "intermediate", (3) "open", and (4) "intermediate". Oscillator strengths are indicated for the most important transitions. Numbers in parentheses give the sum of oscillator strengths in the 300–400-nm region.

teracting but nonconjugated moieties: a tripyrrinone BCD and an amide A.

If one assumes the structures put forward by Rüdiger and co-workers to be correct, the crucial question then is how the far-red band arises in the native P_{fr} form. There is no mechanism to suggest that conjugation between ring A and the rest of the molecule is reestablished in the native form. A possibility to be envisaged, indirectly also supported by spectroscopic investigations on phytochrome-like model compounds,[19-21] is the formation of an anion,[13-18] stabilized by the apoprotein. Dissociation of a proton is known to produce red shifts in pyrrolic systems in general.[24]

III. Computations and Results

Following the lines of previous work,[1,2] we characterize different conformations of the model chromophores by the values of dihedral angles δ_1–δ_6, as defined in Figure 4.[1,2,25] The computation of excited states follows the usual SCF-single excitation–CI scheme, within the frame of an adapted PPP approximation, assuming local σ–π separability. Semiempirical parameters and details on the computation of f values and rotatory strengths have also been previously reported.[1,2]

In the P_r chromophore, assumed to be represented by the structure of Figure 3 excluding all side groups, the nitrogen atom N-1 (Figure 4) is considered to be of "pyridine type," the nitrogen atoms N-7, N-13, and N-19 to be of "pyrrole type."[2]

Three conceivable forms of the P_{fr} chromophore are investigated: (a) a neutral structure (In the absence of conjugation between rings A and B, the electrostatic interaction between the tripyrrinone moiety B–C–D and the amide moiety A′ of ring A is accounted for in the PPP-type approximation by the γ integrals only. We represent this structure as A′· · ·(B–C–D).); (b) a monoionic form in which the proton originally bound to N-13 has been dissociated making the tripyrrinone moiety an anion, schematically A′· · ·(B–C–D)$^-$; (c) a diionic form similar to (b), but in which, in addition, the proton bound to N-19 of the amide moiety has been dissociated, leading to the situation represented schematically as (A′)$^-$· · ·(B–C–D)$^-$. (Although general chemical evidence speaks against this dianionic form, especially in the denaturated pigment, the question of the occurrence of such species has nevertheless been raised in the literature.)[19]

Both forms (b) and (c) should of course be stabilized by the protein. A question of immediate importance is how to simulate the anionic forms in the frame of our semiempirical computations. The number of chromophore electrons remains the same, but the charge of the core is reduced by one unit for each dissociating proton. The nitrogen atoms affected may thus be considered to carry a formal core charge of only +1, although they each contribute two electrons to the chromophore. Their effective valence-state ionization potential I' corresponds to the formal process $N^- \rightarrow N^+ + 2e$. It is assessed within the frame of the PPP approximation and based on previous parametrizations[1,2] in the following manner:

$$I'(N \rightarrow N^+) - EA(N^- \rightarrow N) = \gamma_N = 12.27\ \text{eV}$$

$$I'(N \rightarrow N^+) = 13.40\ \text{eV}$$

By subtraction we find for the valence state electron affinity $EA(N^- \rightarrow N) = 1.13$ eV, from which follows

$$I'(N^- \rightarrow N^+) = EA(N^- \rightarrow N) + I'(N \rightarrow N^+) = 14.53\ \text{eV}$$

As in previous instances, we set[1,2]

$$I'(N \rightarrow N^{2+}) = 19.60\ \text{eV}$$

A test calculation on indole (see Table I) gives satisfactory agreement with experiment.

Table I. Comparison of the Experimental and Theoretical Data for the Longest Wavelength Transition in Indole

	λ, nm	
	neutral	anion
exptl[24]	270 (ϵ_{max} ~5000)	288.5 (ϵ_{max} ~4300)
PPP type	277 (f = 0.036)	285 (f = 0.051)
CNDO[a]	270 (f = 0.158)	287 (f = 0.078)
CNDO[b]	255 (f = 0.175)	280 (f = 0.095)
CNDO[c]	369 (f = 0.071)	386 (f = 0.038)

[a] Parametrization I, ref 2 and 26. [b] Parametrization II, ref 26 and 27. [c] Parametrization III, ref 28.

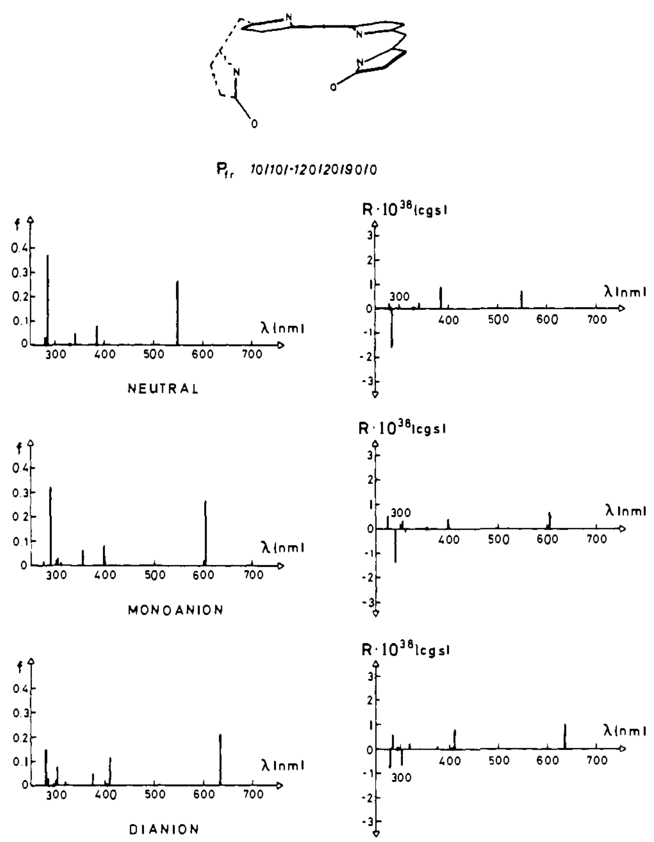

Figure 6. Computed UV-vis and CD spectra of P_{fr} models in a "closed-relaxed" conformation, with carbonyl oxygens separated by ~4 Å. From top to bottom: A′· · ·B-C-D, A′· · ·(B-C-D)$^-$, A′$^-$· · ·(B-C-D)$^-$.

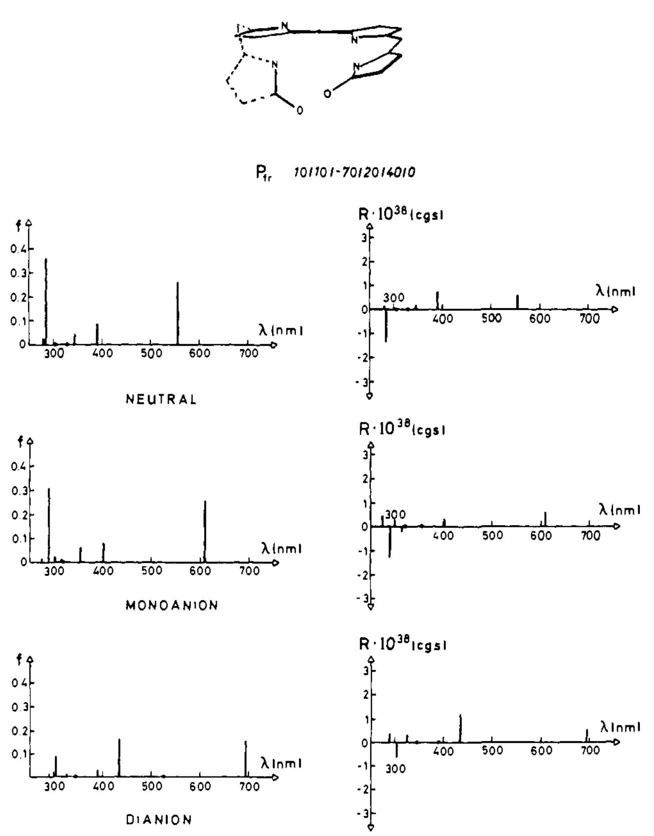

Figure 7. Computed UV-vis and CD spectra of P_{fr} models in a "closed-crowded" conformation, with carbonyl oxygens separated by 2.9 Å (sum of van der Waals radii). One notices (compare Figure 6) that only the spectrum of the dianion is very sensitive to small changes in the relative position of the amide moiety (ring A).

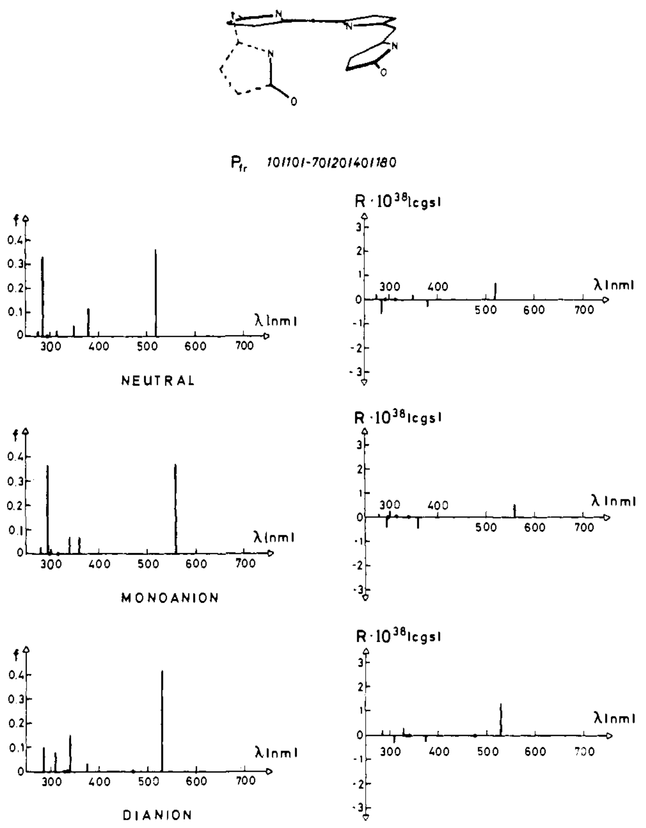

Figure 8. Computed UV-vis and CD spectra of P_{fr} models showing the effect of isomerization around the 6-8 bond.

The infinity of possible conformations $\delta_1/\delta_2/\delta_3/\delta_4/\delta_5/\delta_6$ for P_r and P_{fr} may for our purposes be classified as follows. First are conformations which from previously established spectral rules[1,2,6] or on steric grounds are very unlikely. From the ratio of the intensity of the blue band to that of the red band (f_2/f_1), extremely "closed" or "ring-like" conformations with all δ_i near 0°, or extremely "open" conformations with all or many δ_i near 180°, do not seem likely. Such extremes may also be ruled out by steric arguments, since in very tight "ring-like" structures the terminal chromophore atoms will interfere, and in extremely "open" conformations the binding to a possibly coiled protein might become difficult. Second are conformations which may be viewed as plausible. After performing calculations on about 100 of these, we have picked out some of those few conformations which reproduce the observed chiroptic data in the most consistent fashion.

Computed results are represented in Figure 5 for P_r in (1) a relatively "closed" or "ring-like" conformation with dihedral angles −10/−10/−20/−20/0/0°, (2) an "intermediate" conformation 0/0/−10/−170/−10/+10°, (3) an "open" conformation 170/170/−10/−10/−10/−10°, and (4) an "intermediate" conformation −10/−10/−20/−20/180/180°.

The conformations examined for P_{fr} (Figures 6-8) are closely related to the ones just described for P_r. However, the carbon bridge between rings A and B being saturated, several bond angles have changed. This influences the dihedral angles δ_3 and δ_5. Rotation about the corresponding bonds also is less hindered than in P_r.

As a test for the consistency of our results, we have also performed CNDO-CI-type calculations on the "intermediate" conformation −10/−10/−20/−20/180/180° of the P_r model and on the anion of conformation 10/10/−70/20/40/0° of the P_{fr} model. All adopted parametrizations[26-28] reproduce the red shift in indole upon deprotonation at the nitrogen with

Table II. Optical Properties of P_r and P_{fr} Models Computed in the CNDO Approximation[a]

model	conformation	λ, nm	f	$R \times 10^{38}$, cgs	assignment
P_r	−10/−10/−20/−20/180/180°	509	0.348	−4.55	red band
		360	0.048	−0.54	
		319	0.701	+5.44	blue band
		307	0.005	−1.21	
		298	0.008	0.05	
		283	0.159	−1.09	
P_{fr}	10/10/−70/20/40/0°	538	0.459	+1.52	far-red band
		416	0.024	−0.09	
		344	0.123	+0.61	blue band
		343	0.018	−0.82	
		323	0.022	−0.33	
		322	0.093	−0.06	

[a] With parametrization III: see Table I.

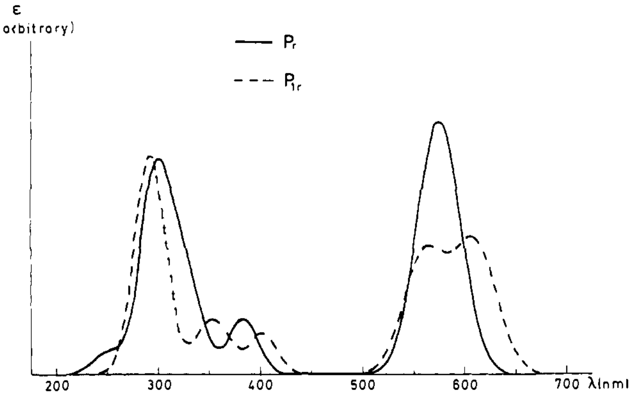

Figure 9. Simulated UV-vis spectra of P_r (—) and P_{fr} (- - -), with P_r in the "intermediate" conformation −10/−10/−20/−20/180/180°. The spectrum of P_{fr} is the superposition of the spectra of the monoanions in the conformations 10/10/−70/20/40/0° and −10/−10/70/−20/−40/180°. Band half-widths are taken as 20 nm, except for the longest wavelength transition, where 30 nm was assumed. For P_{fr} we have weighted the component spectra in the inverse ratio of the longest wavelength oscillator strengths to achieve equal height in the double peak.

roughly comparable accuracy (see Table I). We chose to perform the phytochrome calculations with the parametrization III (Table I) in order to minimize the tendency of our computed absolute wavelengths to appear too far to the blue.

Simulated UV and CD spectra, based on the PPP-type results, are depicted in Figures 9 and 10, respectively.

IV. Discussion

The criteria of comparison with experiment are the wavelengths of the transitions λ_i, the relative oscillator strengths f_i, and the relative rotatory strengths R_i. The computed longest wavelength transitions lie in general about 80 nm (2000 cm^{-1}) too far to the blue and this can be rationalized only partially on the grounds of the neglect of unsaturated side chains. Undoubtedly, the protein plays some role in fixing the position of the longest wavelength transitions.[13] We must stress the fact that the particular PPP-type approximation used here has not been parametrized ad hoc, but rather on simple benzene derivatives,[1,2,29] and one must also bear in mind that slight deviations in wavelength in the 300-nm region of the spectrum are enormously magnified in going to the 700-nm region. The more sensitive criteria for assessing conformations are, therefore, the relative oscillator and rotatory strengths,[1,2,6] as also supported by recent crystallographic evidence.[30] A feature of PPP-type calculations is the appearance of multiple transitions in the 300–450-nm region,[1,2] which are not directly discernible in the experimental spectra.[3] In order to estimate the oscillator strength f_2 of the blue band, the computed values for the transitions within this range were added (values in parentheses in Figure 5). Alternatively, allowing for the possibility that these multiple absorptions are artifacts of the method,[2] we consider only the most prominent transition in this region.

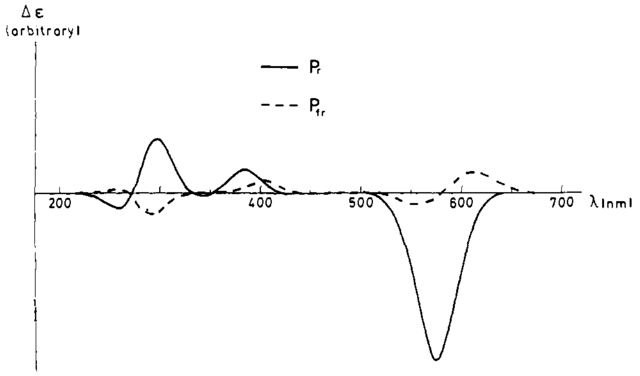

Figure 10. Simulated CD spectra of P_r (—) and P_{fr} (- - -). For details see caption to Figure 9.

Based on the ratio of oscillator strengths f_2/f_1 and on the rotatory strengths R_1, R_2, we conclude that native P_r may exist in conformations of the type 0/0/−10/−170/−10/+10° and −10/−10/−20/−20/180/180°. If one merely considers the relative oscillator strengths, conformation 170/170/−10/−10/−10/−10° agrees best; however, the absolute rotatory strengths are too small.

From the spectrum of denaturated P_r[6] a "ring-like" conformation, such as −10/−10/−20/−20/0/0°, appears to be preferred, thus confirming the assumption[13,22] that unfolding of the protein chain likewise causes drastic conformational changes of the chromophore. A small red shift of the longest wavelength band of P_r upon denaturation is also correctly reproduced by the calculation (Figure 5).

The experimental red shift on going from native P_r to native P_{fr} is about 1260 cm^{-1}. We compare this with the computed shift between P_r in the conformation −10/−10/−20/−20/180/180° (Figure 5) and the monoanion A′···(B–C–D)$^-$ in the conformations 10/10/−120/20/90/0° (Figure 6) and 10/10/−70/20/40/0° (Figure 7): in the first case we obtain a relative red shift of 890 cm^{-1}, in the second case of 1020 cm^{-1}. The sensitivity of this effect to conformation is shown in the case of 10/10/−70/20/40/180° (Figure 8), where a blue shift of 440 cm^{-1} occurs with respect to P_r. Furthermore, we notice that nowhere does a double peak appear in the red region of the spectrum. This is in agreement with experimental data which suggests[7,8,31] that the $P_r \rightarrow P_{fr}$ conversion is incomplete and that a detectable fraction of P_r remains. Consequently, the 660-nm peak corresponds to a residual P_r absorption. An alternative explanation, suggested by our calculations, would be the presence of an equilibrium between different conformers of P_{fr}, like 10/10/−70/20/40/0° and −10/−10/70/−20/−40/180°.

While the neutral form of P_{fr} may definitely be ruled out, the dianionic form is spectroscopically of some interest. One notices in the conformations 10/10/−120/20/90/0° and 10/10/−70/20/40/0° a further red shift of the long-wavelength band, as compared to the monoanion, while interestingly in 10/10/−70/20/40/180° a blue shift occurs. Relative to the computed spectrum of P_r, however, the red shifts are rather too large.

The CNDO-CI results (Table II) do not significantly alter our semiquantitative conclusions. Although the computed absolute wavelengths in general are too short, relative shifts seem to be predicted consistently. In P_r we compute the gap between the red and the blue band to be ~11 700 cm^{-1}, comparing favorably with the experimental value of ~11 000 cm^{-1}. While the observed R_2/R_1 ratio is well reproduced by the calculated conformation (−10/−10/−20/−20/180/180°), the f_2/f_1 ratio is too large, as compared to experiment. This implies that the CNDO result favors the more open conformations for P_r.

The red shift observed upon transformation to P_{fr} is computed to be 1060 cm^{-1}, as compared to 1260 cm^{-1} experimentally. Both the sign change and the absolute weakening of the long-wavelength Cotton effect with respect to P_r are reproduced by the calculation as well. The computed absolute R value seems somewhat too large. The oscillator strength ratio f_2/f_1 indicates that in this case CNDO- and PPP-type calculations agree in preferring the relatively closed conformation (10/10/−70/20/40/0°) for P_{fr}. A double transition in the red–far-red region is again absent, leading to the assumption that the observed double peak may be due to an equilibrium between different species and (or) conformations.

Assuming the proposed structures[13,18] to be correct, we therefore conclude that the phototransformation $P_r \rightleftharpoons P_{fr}$ must involve (1) the dissociation of (at least) one proton from the P_r chromophore; (2) the adoption of a more "closed" conformation in P_{fr} relative to P_r, with at least partial inversion of chirality; (3) the establishment of an equilibrium between P_r and P_{fr} and/or between different conformations of P_{fr}. This would presumably induce changes in the immediate protein structure as well, thereby stabilizing the converted chromophore.

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