# *CONFORMATION DEPENDENCE OF INTRAMOLECULAR ENERGY TRANSFER IN PHYCOERYTHRIN**

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*Communicated by Lawrence R. Blinks, November 16, 1967*

The strongly fluorescent, water-soluble, photosynthetic accessory pigment, phycoerythrin, that colors red algae and that has classically served in the development of techniques for separating proteins is of special interest in intramolecular energy transfer because several different chromophores are covalently bonded as residues along its polypeptide chain. More specifically, nearly 5 per cent of the protein is in tyrosyl residues,[1] and of the 7 per cent pigment[1] in one molecule of R-phycoerythrin I[2] there are about 37 phycobilins, 25 of which are phycoerythrobilin (PEB) and 12 of which are the more stable isomer of oxidized PEB, phycourobilin (PUB).[3] PEB residues in two different intramolecular environments are responsible for the 540-m$\mu$ ($PEB_{540}$) and 565-m$\mu$ ($PEB_{565}$) absorption maxima of phycoerythrin, and the 500-m$\mu$ peak is due to PUB.[4] The fluorescence spectrum of phycoerythrin has a sharp maximum at about 580 m$\mu$ and a shoulder or secondary maximum near 630 m$\mu$.[5] The $PEB_{565}$ residues, probably 15 of them,[6] are solely responsible for this fluorescence which is typically lost together with $PEB_{565}$ absorbance upon denaturation.[4] Evidence for independent PUB and PEB chromophores was obtained by the reappearance of fluorescence in two bands after the formation of a zinc complex with denatured phycoerythrin.[7] Action spectra for the fluorescence of the native phycoerythrin have shown that electronic excitation energy of the accessory chromophores is transferred to $PEB_{565}$, but that of tyrosyl is not transferred efficiently.[8-10] At the higher, intermolecular order of integration phycoerythrin is a photosynthetic accessory pigment whose excitation energy is probably transferred by inductive resonance[11] first to phycocyanin, then on chiefly to a bed of chlorophyll *a* that sensitizes the catalyst of photosystem II and partly to the long-wavelength, fluorescence-quenched chlorophyll *a* of System I.[12, 13] Strong interactions of chlorophyll were indicated in chloroplasts by means of optical rotatory dispersion (ORD),[14-16] which has some repute in testing protein conformation and chromophore interaction.[17, 18] The techniques of fluorescence spectroscopy and ORD were combined in the present work to examine intramolecular energy transfer as a function of conformation in phycoerythrin.

*Methods.*—R-phycoerythrin I was extracted from *Antithamnion* sp. by Prof. L. R. Blinks at Pacific Grove, California, precipitated from half-saturated ammonium sulfate, redissolved in 0.05 *M* sodium phosphate buffer pH 7.2 (batch 1), or repeatedly reprecipitated and redissolved in distilled water (batch 2). The solutions were stored at 4°C in the dark for no more than 10 days. Only the proportion of visible to ultraviolet absorption differed between batches of final solution (Figs. 1*A* and 2*A*). Batch 1 was examined in the ultracentrifuge by Dr. Hiroshi Mizukami and it displayed only one peak which was symmetrical and yielded a sedimentation constant ($s_{20,w}$) of $11.6 \times 10^{-13}$ cm/sec in agreement with Eriksson-Quensel.[19]

Absorption spectra were measured on Cary spectrophotometer models 11 and 15,

Optical rotatory dispersion was measured with a Cary model 60 spectropolarimeter. Fluorescence was measured with a sensitive spectrofluorimeter in which light from a 900-w Xenon arc was diffracted by one or two Bausch & Lomb 250-mm model monochromators, chopped at 200 cps and focussed on a 1-cm-square fused quartz fluorescence cell. The light emitted 90° from the incident beam was analyzed by passage through a Bausch & Lomb 500-mm monochromator and measured with an EMI 6255B photomultiplier tube whose signal was amplified by a Princeton Applied Research model JB4 lock-in amplifier and recorded on a Mosely Autograf X-Y recorder against the wavelength of the exciting or emitted light. The recorder was synchronized with the wavelength drum of the monochromator being used for variable wavelength. Fluorescence spectra were manually corrected for the spectral sensitivity of the photomultiplier and for the spectral efficiency of the analyzing monochromator. Action spectra for fluorescence were similarly corrected for the variations in incident-light energy measured with a thermopile, kindly lent by Prof. R. S. Livingston. Actual fluorescence yields were not measured and the spectra were interpreted on the basis of the relative fluorescence intensities in arbitrary units.

Concentrations were determined with Ó Carra's specific extinction coefficient.[2] A 1.5-ml sample of $5.3 \times 10^{-7}$ *M* phycoerythrin was bleached by irradiation with white light of 0.04 w/cm$^2$ intensity while thermostatted at 5°C for 15 hr. Phycoerythrin was denatured by standing in 8 *M* or 4 *M* urea solution for 48 hr at 4°C. PEB was removed from phycoerythrin by two methods as described by Ó Carra *et al.*[3]

*Results.—Native phycoerythrin:* The characteristic absorption maxima of the native phycoerythrin were consistently observed here at 278, 308, 370, 497, 538, and 566 mμ (Figs. 1*A* and 2*A*). The optical rotation (Fig. 1*B*) was very large in the ultraviolet where there was a Cotton effect, with a pronounced negative trough at 232 mμ, characteristic of the peptide chromophore of the protein moiety. There was also a strong positive Cotton effect corresponding in wavelength to the $PEB_{566}$. Weaker rotatory power appeared to be associated with the absorption of $PEB_{538}$ and $PUB_{497}$. An acute minimum at 311 mμ may be construed to be a result of interference of a negative Cotton effect by another absorption band contributing to the 308-mμ absorption maximum. To remove the involvement of protein-prosthetic group coupling, the pigment was bleached by light and its ORD was reexamined. As a result only the absorption maximum of shortest wavelength remained, shifted down by about 8 mμ (Fig. 1*A*). Similarly only the protein's Cotton effect remained, but it was much diminished (Fig. 1*B*).

The native phycoerythrin fluoresced maximally at 578 mμ. The fluorescence spectrum (Fig. 1*C*) was scanned from 250 mμ to 600 mμ and only the one peak was evident. The same fluorescence spectrum was obtained when the sample was illuminated with monochromatic light corresponding to each of its absorption maxima, including that at 278 mμ. The fluorescence action spectrum (Fig. 1*D*) showed maximal emission of light absorbed in the range 480–550 mμ by $PUB_{497}$ and $PEB_{538}$, and lesser excitation maxima appeared at 378 mμ and 302 mμ which approximated two other absorption maxima. Light of wavelength 278 mμ produced some long-wavelength fluorescence but did not elicit a peak in intensity of that emission. At the red end also there was no emission maximum corresponding to $PEB_{566}$, and exciting light of wavelength 566 mμ had 0.8 of the effect of 538-mμ light.

*Denatured phycoerythrin:* Denaturation by 8 *M* urea decreased the absorbance of phycoerythrin in the visible range and increased that in the ultraviolet (Fig.

2A). The PEB residues, particularly $PEB_{566}$, were especially affected such that the absorbancies of denatured phycoerythrin at the maxima 497 mμ, 538 mμ, and 566 mμ were 64 per cent, 32 per cent, and 22 per cent, respectively, of those of native phycoerythrin. Denaturation by 4 *M* urea resulted in corresponding decreases to 76 per cent, 58 per cent and 51 per cent, respectively (Fig. 1A), but the

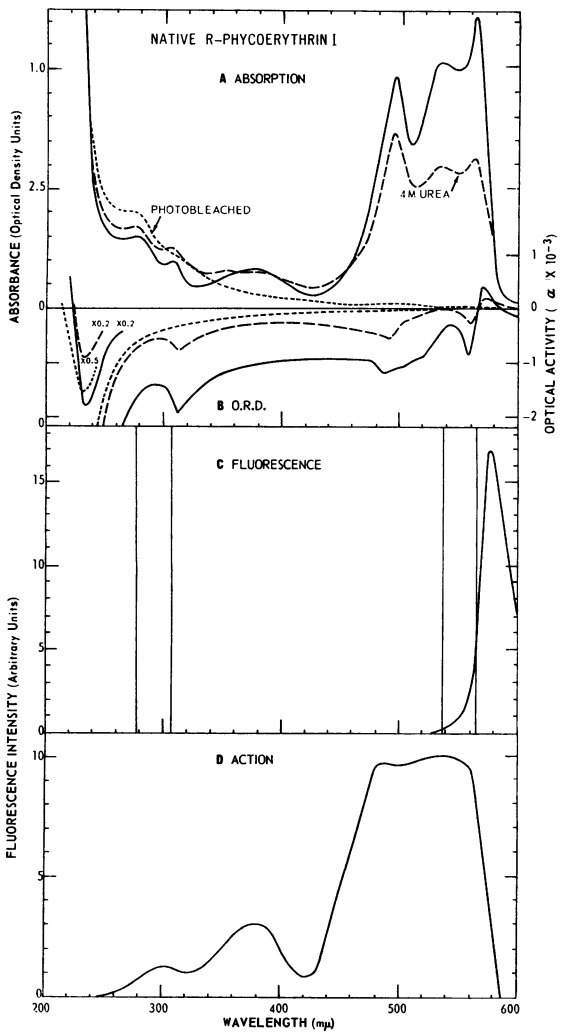


FIG. 1.—Spectral properties of native phycoerythrin:

(*A*) Absorption spectrum of a solution of $5.3 \times 10^{-7}$ *M* phycoerythrin in distilled water (*solid line*), of the same in 4 *M* urea (*dashed line*), and of a photobleached sample in distilled water (*dotted line*).

(*B*) Specific rotation of above solutions except native phycoerythrin in 0.05 *M* phosphate buffer pH 7.2.

(*C*) Fluorescence spectrum of $4.4 \times 10^{-7}$ *M* phycoerythrin in water in 479 ± 3 mμ exciting light, typical of excitation with wavelengths indicated by vertical lines. Resolution 3.3 mμ.

(*D*) Action spectrum for fluorescent light emitted at 589 ± 2 mμ by $4.4 \times 10^{-7}$ *M* phycoerythrin in water. Exciting band width, 1.3 mμ.

absorbance at 566 mμ was not diminished as much as the Cotton effect (Fig. 1B). $PEB_{566}$ was still spectroscopically evident in 8 *M* urea which completely abolished the Cotton effect at this wavelength (Fig. 2B). The rotatory effect at 497 mμ was not influenced by the urea solutions, but the short-wavelength absorption by 8 *M* urea occluded the peptide Cotton effect.

The fluorescence of the denatured phycoerythrin was weak and its spectrum

was dependent on the exciting wavelength. The several spectra in Figure 2*C* are not to be compared with respect to intensity of fluorescence, as different uncalibrated instrumental sensitivities were used in obtaining them. Illumination with light of wavelength 278 mμ which corresponded to the ultraviolet absorption maximum of phycoerythrin caused the denatured phycobiliprotein to fluoresce with maxima at 299 mμ and 359 mμ (Fig. 2*C*, *solid line*). The instrument was

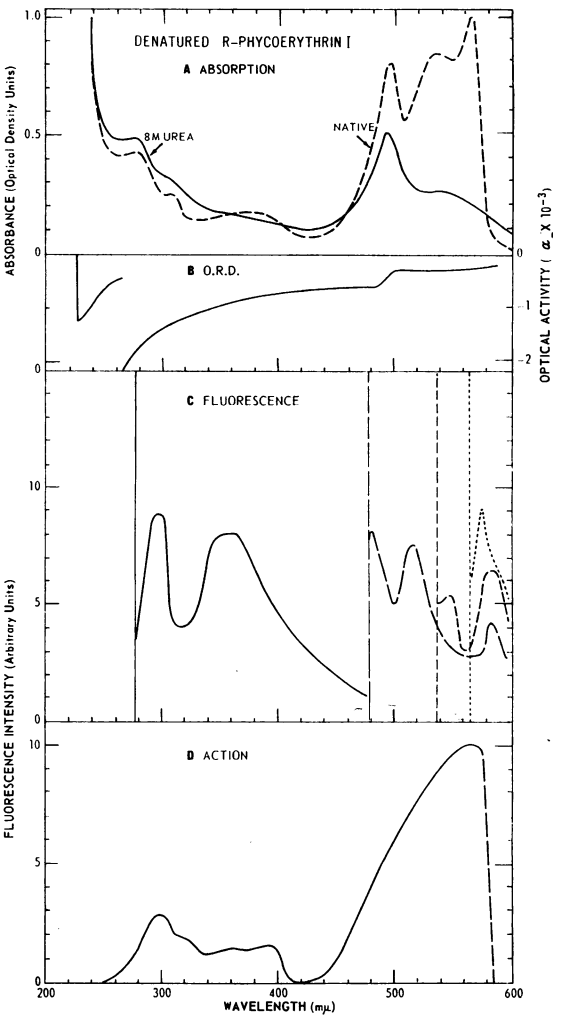


FIG. 2.—Spectral properties of denatured phycoerythrin.

(*A*) Absorption spectra of 4.4 × $10^{-7}$ *M* phycoerythrin both native in water (*dashed line*) and denatured in 8 *M* urea (*solid line*).

(*B*) Optical rotatory dispersion pattern of the denatured preparation (UV data ×0.2).

(*C*) Fluorescence spectra of the denatured preparation resulting from excitation with 7-mμ bands of light of wavelengths 278 mμ (*solid lines*), 479 mμ (*broken lines*), 538 mμ (*dashed lines*), and 566 mμ (*dotted lines*). Resolution, 1.7 mμ.

(*D*) Action spectrum for fluorescence of the denatured preparation measured at 589 ± 2 mμ. Exciting band width, 3.3 mμ.

not sufficiently sensitive to extend this spectrum reliably to longer wavelengths, but there were indications of rises in emission above the noise near 520 mμ and 585 mμ. In light of wavelength 479 mμ, the denatured phycoerythrin yielded a fluorescence spectrum with a maximum at 519 mμ and evidence for a minor peak at 585 mμ, but stray exciting light may have been at least partly responsible for the high readings at 482 mμ (*broken line*, Fig. 2*C*). Monochromatic excitation at

538 mμ resulted in the production of a fluorescence maximum at 550 mμ and also at about 585 mμ (*dashed line*, Fig. 2*C*). The long-wavelength maximum of fluorescence was resolved to 578 mμ when the denatured phycoerythrin was illuminated with light of wavelength 566 mμ (*dotted line*, Fig. 2*C*).

The action spectrum of the long-wavelength fluorescence of the denatured phycoerythrin (Fig. 2*D*) resembled that of the native phycoerythrin. However, the excitation at 300 mμ was more effective than that at the next maximum, at 360–390 mμ, and there was only one maximum in the visible part of the spectrum, near 570 mμ. This action spectrum was obtained with high instrumental sensitivity, as the fluorescence yield was considerably lower than that of native phycoerythrin.

*Phycobilins:* The absorption spectrum of a solution of ether-soluble PEB in chloroform, prepared by the preferred method of Ó Carra *et al.*,[3] included absorption shoulders at 255 mμ and 300 mμ and a single maximum in the visible range at 502 mμ in agreement with the spectrum of the free base in chloroform.[3] Such a preparation has been reported previously to be nonfluorescent. However, we recorded distinct fluorescence at shorter wavelengths than suspected, with maximum emission at 372 mμ or at 334 mμ for the acid form. This short-wavelength fluorescence was also displayed by water-washed, chloroform-soluble pigment from the acid hydrolysate, in which visible absorption was practically abolished, leaving an absorption maximum at 275 mμ in chloroform or dilute acid (Fig. 3).

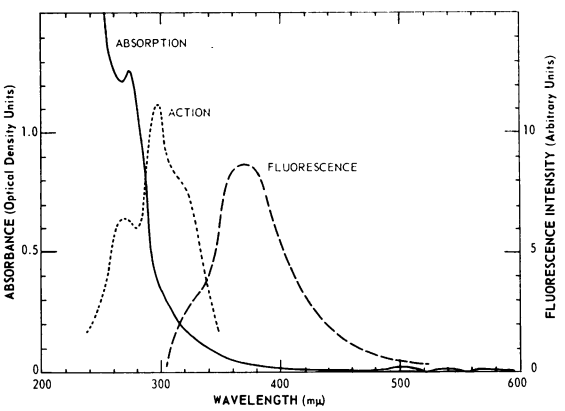


FIG. 3.—Spectral properties of a degraded phycobilin extract in chloroform. Absorption spectrum (*solid line*). Fluorescence spectrum, with 10 mμ resolution, from excitation at 281 ± 7 mμ (*dashed line*). Action spectrum with 13-mμ resolution for fluorescence at 372 ± 5 mμ (*dotted line*).

The most effective exciting wavelength for this fluorescence was 300 mμ in chloroform (Fig. 3) or 286 mμ in acid, and a secondary peak occurred at 270 mμ in both solutions. No optical activity was detected by measurements of ORD.

*Discussion.*—The outstanding rotatory strength of the $PEB_{566}$ residues and its loss by denaturation confirmed Ó hEocha and Ó Carra's[7] conclusion that $PEB_{538}$ and $PEB_{566}$ differ by virtue of their intramolecular environments, and they supported Teale and Dale's[6] conclusion from measurements of fluorescence polarization that the protein structure imposes on fluorescent PEB a nonrandom specific orientation. By its ability to fluoresce, $PEB_{566}$ corresponds to the tyrosyl residue of Type Ib in Cowgill's classification,[20] which is an isolated buried residue. The

lack of an effect of denaturation on the ORD contribution of $PUB_{497}$ indicates that, unlike $PEB_{566}$ or to a lesser extent $PEB_{538}$, its environment was not changed by the unfolding of the protein. This agrees with Ó hEocha's[4] deductions that phycoerythrobilin residues are located in the hydrophobic region of the biliproteins and that phycourobilin residues are not masked to the same extent. Abolition of the Cotton effect of $PEB_{566}$ by 8 *M* urea showed that the bonding situation which induced that optical activity was the result of three-dimensional folding or arrangement of subunits, as shown with model compounds.[21] Tight bonding[22] or the asymmetrical helix[23] of PEB in a hydrophobic environment[24] may be responsible. The Cotton effect of the $PEB_{566}$ was positive, as was that corresponding to the red absorption band of chlorophyll whose dimerization is promoted in solution or in chloroplasts.[16] We have not examined solutions of PEB to rule out the possibility of a similar induction of optical activity in it, such as is shown to exist for dinucleotides.[25] The start of a negative Cotton effect above 311 mμ was also reminiscent of the reversed position observed at the blue absorption maximum of chlorophyll in dilute solution.[14, 16] Since PEB in the acid form has an absorption maximum at this wavelength,[3] the activity may be related to the bound $PEB_{566}$.

Considering the peptide Cotton effect, the value of the reduced mean residue rotation ($m'_{232}$) computed therefrom was changed roughly 4000 units by denaturation. This indicated a higher helix content than the 15 per cent helix estimated for chloroplast lamellar protein,[15] but the extrinsic Cotton effects caused by the couplings of the prosthetic groups made calculations dubious. It was hoped that photobleaching would remove these effects and it did indeed, but it also strongly removed any ordered structure that might have existed and appeared to denature phycoerythrin more effectively than did 8 *M* urea. The increased optical density in the ultraviolet was also probably indicative of protein denaturation.[26]

The strong fluorescence and absorbancy of $PEB_{566}$ in native phycoerythrin, in which the residues were in close proximity, was typically lost during the unfolding in denaturing agents. In 8 *M* urea, interchromophore energy transfer was disrupted and a fluorescence maximum was detected for each absorption maximum tested for this property. The fluorescence of the individual chromophores is considered a result of impairment of energy transfer by increased distances between the chromophores bonded as residues to the protein. Light-excited $PUB_{497}$ and $PEB_{538}$ fluoresced separately but also sensitized the fluorescence of some remaining $PEB_{566}$. Light absorbed by the tyrosyl band[1, 8, 9, 27] was in part directly emitted at 299 mμ. Dale and Teale[9] also noted an increased ultraviolet fluorescence in bleached phycoerythrin, and at least heat denaturation is known to free intramolecularly bound tyrosine so that it will fluoresce.[28] Tyrosine fluoresces at 304 mμ in insulin and ribonuclease.[28] The excitation energy of tyrosyl in our denatured phycoerythrin was partly transferred, then re-emitted at 359 mμ. The chromophore so sensitized probably absorbed maximally at the 308-mμ peak. Since observations of the effects of denaturation on this peak in all spectra were analogous to those at 566 mμ, and since a similar fluorescence maximum resulted from the excitation of phycobilin extracts with light of about 300 mμ, it seems

likely that this spectral component of phycoerythrin is a small, relatively stable chromophore in the phycobilin moiety rather than a different residue.

Even in native phycoerythrin, energy transfer from the tyrosyl residue to the visibly fluorescing chromophore $PEB_{566}$ was inefficient, which is in agreement with information since published by Eriksson and Halldal[8] and contrary to Bannister's results with phycocyanin.[27] However, our action spectra and also the fluorescence spectra with 278-m$\mu$ excitation showed that some such energy transfer did occur. Probably most but not all of the tyrosyl residues were incapable of resonance energy transfer as a result of hydrogen bonding to carboxylate groups in hydrophobic regions of the native protein.[20, 28]

Considering the action spectra further, the visibly absorbing accessory chromophores in native phycoerythrin sensitized the long-wavelength fluorescence more effectively than did the 566-m$\mu$ and 308-m$\mu$ absorption bands, in contrast with their contributions to the absorption spectrum. Denaturation reversed these relationships. The decreased fluorescence efficiency of native phycoerythrin at the red end of the spectrum has been reported previously with reservations concerning the measuring technique.[8] Because effective red sensitization of fluorescence was in fact observable here, we conclude from the lack of it in native phycoerythrin that the fluorescence of some of the $PEB_{566}$ residues was quenched. The decrease in total PEB during denaturation is attributable to its well-known lability, and the associated appearance of fluorescence at 359 m$\mu$ may be related to that property in extracts of degraded phycobilins. The removal of the Cotton effect and of the quenched fluorescent fraction of $PEB_{566}$ by denaturation suggests that some $PEB_{566}$ residues were intramolecularly hydrogen-bonded into helices[24] or dimerized[29] within the native apoprotein.

The evidence for the interresidue resonance transfer of energy, for the special binding in hydrophobic regions of the residues that constitute the long-wavelength energy sink, and for the accompanying "red drop" in fluorescence efficiency bears a striking analogy to the picture of energy transfer at the intermolecular level in the photosynthetic system.

*Summary.*—In the native state of R-phycoerythrin I, energy was transferred very efficiently between its several chromophores excepting tyrosyl, and a "red drop" in the action spectrum for fluorescence indicated a quenched fraction of the long-wavelength form of phycoerythrobilin. This form also contained a helical or firmly bound fraction with a strong Cotton effect, and the phycoerythrobilin residues, unlike those of phycourobilin, were probably buried in the protein as their rotatory power was removed during the unfolding of the protein by denaturation in 8 *M* urea. Denaturation separated the accessory chromophores sufficiently for them to fluoresce independently. This fluorescence included a new band in the near ultraviolet, probably derived from the 308-m$\mu$ absorption band, which was tentatively ascribed to a more stable chromophore in the phycoerythrobilin residue. Photobleaching denatured the protein more effectively than did 8 *M* urea.

We are grateful to Prof. Rufus Lumry for laboratory facilities and helpful encouragement and to Prof. L. R. Blinks for the samples of phycoerythrin.

* This work was aided by grants from the U.S. Atomic Energy Commission (AT (11-1) 794). Paper no. 27, Laboratory for Biophysical Chemistry, University of Minnesota.

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