Chemical Modification of Biliprotein Chromophores *

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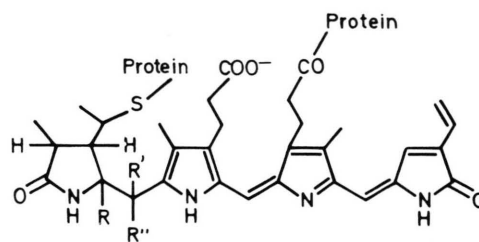
The reaction of biliproteins with sodium dithionite has been studied. The reagent is selective towards the chromophores. In denatured phycocyanin from *Spirulina platensis,* all three chromophores react to form yellow "phycorubin", whereas only 1/3 of the chromophores react in native phycocyanin in a non-statistical manner. From reversion experiments, it can be shown, that the thermodynamic stability of the chromophores towards reaction with dithionite is increased in the native pigment. Similarly, native phytochrome in its P_r form reacts only partially to a pigment absorbing at both 420 and 660 nm. The same product is formed from native P_{fr}, indicating both a reversion to P_r and a partial reduction.
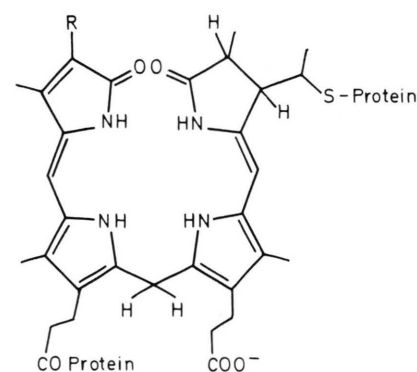
Introduction

Biliproteins are chromoproteins containing bile pigment chromophores (A-dihydrobilins [2 − 6], *e. g.* formulas 1, 2) *covalently* linked via a thioether bond to the apoprotein [7 − 10]. The *phycobiliproteins* (phycocyanins (PC), allophycocyanins and phycoerthyrins) are the main light harvesting pigments of photosystem II of the blue-green, red and cryptophytan algae [11 − 13]. As has been shown by denaturation studies, the physical and chemical properties of the phycobiliprotein chromophores, which fit them for their biological functions, are profoundly influenced by *noncovalent interactions* with the protein moieties ("molecular ecology"). Native phycobiliproteins are highly fluorescent and transfer the absorbed energy to the photosynthetically active reaction center chlorophyll by the mechanism of induced fluorescence (see [14] for references). This fluorescence is completely quenched by denaturation [15]. Also, the main absorption band for denatured phycobiliproteins is in the near uv spectral region, but the native forms have their main absorption band in the red spectral region [1], a prerequisite for effective photosynthesis of the algae at their natural habitat. Similarly pronounced differences between the *native* and *denatured* pigments exist in *phytochrome* [16], the photomorphogenetic reaction center pigment of higher plants and some algae [17]. It under-
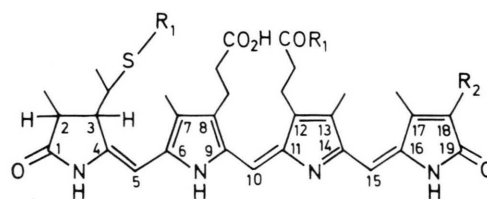
Formula 1:
PC: R_1 = Protein, R_2 = Ethyl
P_r: R_1 = Protein, R_2 = Vinyl

Formula 2: P_{fr}

Formula 3:
Phycorubin: R = Ethyl
Phytochromorubin: R = Vinyl

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goes photochemical conversion from P_r to P_{fr}, the physiologically active form, only in the *native* state. An approach to study the *noncovalent interactions* between chromophores and apoproteins in the biliproteins responsible for the changes cited above more precisely, was made by the method of selective chemical modification of the bile pigment chromophore.

By comparison of the properties of the thus obtained "synthetic" chromophore, when the apoprotein is in its native or denatured form, respectively, a better insight of the influence of the protein moiety on the chromophore is expected. For the first experiment of this type, the reductant dithionite was chosen as a chromophore selective reagent. Interestingly, dithionite (and other reductants) have been shown earlier to accelerate the *in vitro* dark reversion of P_{fr} to P_r [18].

Materials and Methods

PC was isolated from *Spirulina platensis*. The deep-frozen cells (30 g) were broken with glass beads (0.17 mm ⌀) in a cell mill, and the debris was extracted twice with 50 ml tris-buffer (10 mM, pH 8.0). The resulting crude extract was centrifuged at $78{,}000 \times g$ for 60 min. The protein was purified by chromatography on DEAE-cellulose (DE 52, Whatman, England) and subsequent gel filtration on Biogel P 150 (Biorad, California). $E_{620/280} = 4.2$.

Phytochrome was isolated from oat using a modified procedure [19] of the method described by [16]. It had a purity index $E_{280/660} = 20$ and a molecular weight of 60,000. Work with *phytochrome* was done under green safety light.

PC was denatured by adding 200 µl of a stock solution in phosphate buffer to 2.0 ml of urea buffer (8 M urea, 50 mM phosphate pH 7.5), yielding a final concentration of 9×10^{-6} M in phycocyanobilin chromophores. *Phytochrome* (final concentration 3×10^{-6} M in phytochromobilin chromophores, calculated from the extinction coefficient of [20]) was denatured by addition of solid urea (8 M) to its solution in tris-buffer (10 mM, pH 7.4). For reduction, the *denatured* pigments were treated with solid sodium dithionite (5×10^{-3} M), or aliquots of a freshly prepared solution of dithionite in phosphate buffer were added (final concentration 5×10^{-4} M). PC was renatured by removal of urea by gel filtration on a 1.7×12 cm Biogel P 2 column equilibrated with

phosphate buffer. This procedure was carried out either in the absence ("aerobic renaturation") or in the presence of 5×10^{-3} M dithionite ("anaerobic renaturation"). *Native* biliproteins were treated with dithionite as described for the *denatured* forms.

UV-vis spectra were recorded on a DMR 22 spectrophotometer (Zeiss, Oberkochen). The phytochrome measurements were performed with a UVP 300 spectrophotometer (Shimadzu, Japan) equipped with a DBC-2 baseline correction unit in the double-beam mode.

Results

Phycocyanin: If *denatured* PC * was treated with sodium dithionite, the bands at 602 and 355 nm disappeared, and a new band arose at 418 nm (Fig. 1 a). A spectroscopically identical reaction product was obtained, if 5×10^{-4} M or 5×10^{-3} M dithionite were used, but the reaction was faster with the latter concentration (decrease from 20 min to 10 seconds for completion at room temperature).

If the yellow product (denatured, dithionite-treated PC) was *renatured* by removal of urea (gel filtration on biogel P 2) in the absence of dithionite ("aerobic renaturation") (Fig. 1 b) or at a dithionite conc. of 5×10^{-4} M, the blue colour of *native* PC reappeared. The product was identical to native PC as judged from its uv-vis spectroscopic and electrophoretic properties. If the renaturation procedure was carried out in the presence of 5×10^{-3} M dithionite ("anaerobic renaturation", Fig. 1 b), the resulting pigment had absorption bands at both 620 nm and 418 nm ($E_{620}/E_{418} = 3 : 1$). If this greenish-blue pro-
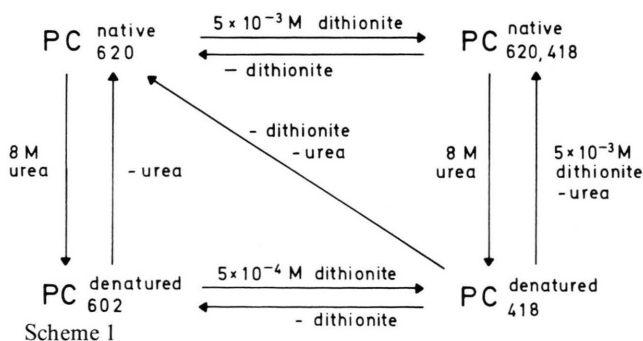
Scheme 1

* The results obtained with PC are summarized in Scheme 1, those for phytochrome in Scheme 2 and Table I. The subscripts for the pigments refer to the approximate maxima of their visible absorption bands, the surscripts to the state of the protein.
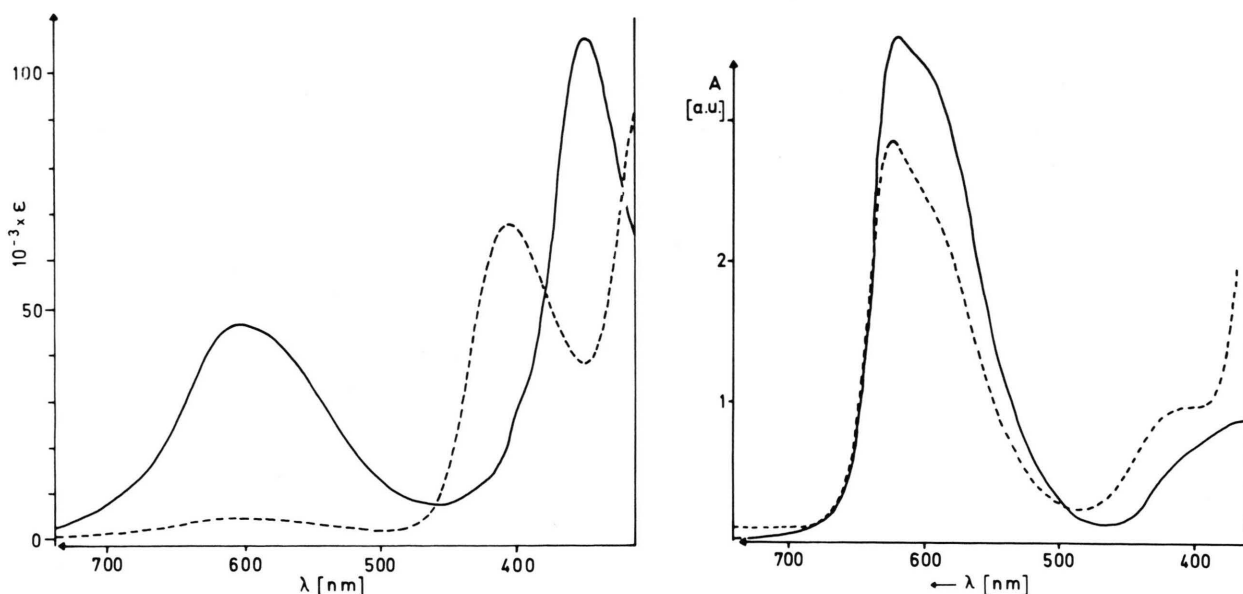
Fig. 1. Uv-vis spectra of the products shown in Scheme 1. a) $PC_{602}^{denatured}$ (————): *Denatured* PC in phosphate buffer (0.05 M), pH 7.5, containing urea (8 M); $PC_{418}^{denatured}$ (— — — —): The same solution after addition of sodium dithionite (5×10^{-3} M). b) PC_{620}^{native} (————): $PC_{418}^{denatured}$, renatured by removal of urea and dithionite by gel filtration. $PC_{620, \, 418}^{native}$ (— — — —): $PC_{418}^{denatured}$ after removal of urea *only* by gel filtration in the presence of dithionite (5×10^{-3} M).

duct was denatured again by addition of solid urea (8 M) without further addition of dithionite, the yellow colour returned immediately, the absorption spectrum showing a single peak in the visible region at $\lambda_{max} = 418$ nm. No bleaching of the 620 nm band was seen, when *native* PC was treated with 5×10^{-4} M dithionite. At a concentration of 5×10^{-3} M, E_{620} decreased by 26% (the integrated intensity of the long wavelength band by 35%) and a peak at 418 nm appeared. Spectroscopically the pigment obtained this way resembled that of the "anaerobic renaturation" procedure.

Phytochrome *: After denaturation of P_r by addition of urea to a final concentration of 8 M, the original band at 660 nm was shifted to 655 nm and de-

Scheme 2

* See footnote p. 3.

creased to 27%. Dithionite treatment (5×10^{-3} M) of the *denatured* P_r resulted in total bleaching of the long wavelength band and the apperance of a band at 422 nm. These changes were also seen in the difference spectrum (P_r *denatured*/5×10^{-3} M dithionite *minus* P_r *denatured*) showing a positive band at 430 nm and a negative one at 655 nm. Denaturation of P_{fr} in the free base form resulted in conversion to P_r, as has been described earlier [16], and therefore, the products resulting from dithionite treatment were identical to those obtained for P_r. Addition of 5×10^{-3} M sodium dithionite to *native* P_r yielded only partial bleaching of the band at 660 nm. The uv-vis difference spectrum (P_r *native*/5×10^{-3} M dithionite *minus* P_r *native*) had negative bands at 660 nm (and 730 nm) and a positive band at 433 nm. From this difference spectrum, a decrease of the original E_{660} by $10 - 15$% was calculated. *Native* P_{fr} showed uv-vis absorption bands at 660 and 720 nm. Upon treatment with 5×10^{-3} M dithionite, the band at 720 nm disappeared, the absorption band at 660 nm increased by about 40%, and a new band was formed at 425 nm, corresponding to a negative band at 720 and two positive bands at 655 and 430 nm in the difference spectrum (P_{fr} *native*/5×10^{-3} M dithionite *minus* P_{fr} *native*) (Fig. 2).
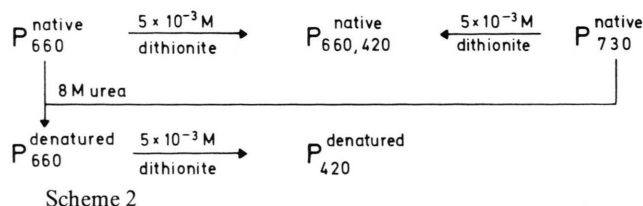
Table I. Uv-vis spectra of the phytochrome reduction experiments with dithionite.

| Compound | Uv-vis absorption spectra | | | | Extrema in the uv-vis difference spectra | Intensity change of the long wavelength bands after dithionite-treatment, calculated from uv-vis absorption and uv-vis absorption difference spectra |
| | Original (I) | | after treatment with 5×10^{-3} M dithionite (II) | | (II minus I) | |
	λ_{max} [nm]	E [a]	λ_{max} [nm]	E [a]	λ_{max} [nm]	
P_r, native	660	0.2	660	0.18	660 neg.	10 – 15% decrease [a]
	730	0.01	b		730 neg.	100% decrease
					433 pos.	
P_r, denatured	655	0.06	422	0.08	655 neg.	100% decrease
					430 pos.	
P_{fr}, native	660	0.07	660	0.1	655 pos.	40% increase
	720	0.06	425	0.06	720 neg.	100% decrease
					430 pos.	

[a] Quantification of the results was difficult for some samples, due to turbidity and bleaching of the background absorption by the dithionite.
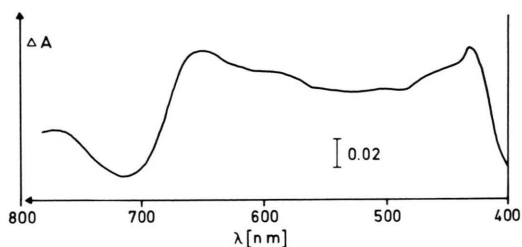[b] At $\lambda < 430$ nm undetermined due to the turbidity of the sample.

Fig. 2. Uv-vis difference spectrum of the reaction of phytochrome P_{fr} with 5×10^{-3} M sodium dithionite. $P_{fr, native}$/sodium dithionite *minus* $P_{fr, native}$.

Discussion

Denatured PC or P_r could be completely converted to yellow pigments with sodium dithionite. Fischer and Plieninger [21] have reported, that biliverdin is reduced by this reagent to bilirubin. A rubinoid pigment ($\lambda_{max} = 420$ nm) has been obtained, too, from phycocyanobilin [22]. The reduction products of PC and P_r had absorption maxima (418 and 422 nm, respectively) as expected for rubins (400 – 460 nm), e. g. [21, 23]. Assuming a similar reduction by dithionite as has been reported for biliverdin [21], an A-dihydro-(meso)-bilirubin structure is suggested for these pigments (formula 3), which we have named *"phycorubin"* and *"phytochromorubin"*.

Treatment of *native* biliproteins led only to partial reaction, even at a dithionite concentration of 5×10^{-3} M, exceeding ten times the concentration

sufficient for complete reduction of the *denatured* forms (5×10^{-4} M). From their uv-vis spectral data, the products obtained at $c_{dithionite} = 5 \times 10^{-3}$ M contain verdin-type chromophores (formula 1) as well as rubin-type chromophores (formula 3). From the decrease of the intergrated intensity of the long wave-length band of 35%, it can be estimated, that about one third of the phycocyanin chromophores are in the reduced state. A non-statistical reduction of the three chromophores present in the PC monomer is supported by the uv-vis difference spectrum (PC *native minus* PC *native*/5×10^{-3} M dithionite), which has a negative double band at 596 and 616 nm (blue-shifted with respect to the absorption maximum of *native* PC at 620 nm), as well as by the fluorescence excitation spectra * of partially reduced PC, which do not exhibit the short wavelength shoulder present at 600 nm in *native* PC. Thus, the chromophores absorbing at shorter wavelengths are preferentially reduced.

Several explanations may principally be given for the different behavior of *denatured* and *native* biliproteins. The protein could shield the chromophores from the aqueous environment and thus prevent the attack of the reductant, or, the activation energy for the reduction of the chromophores could be enhanced in the *native* as compared to the *denatured*

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state. If these kinetic arguments would hold, the yellow chromophores would have to be accomodated by the protein.

However, these *kinetic* arguments are not in agreement with the results of the renaturation of *"phycorubin"*. During renaturation at $c_{\text{dithionite}} = 5 \times 10^{-4}$ M, complete reoxidation occured. At $c_{\text{dithionite}} = 5 \times 10^{-3}$ M ("anaerobic renaturation"), the chromophores were still reoxidized to the most part, and only 20 − 30% remained reduced, if judged by the uv-vis spectrum (Fig. 1 b). The renaturation products thus resemble those obtained by dithionite treatment of the *native* PC at the corresponding concentrations. Any artefact due to possible losses of dithionite by air-oxidation during the chromatography could be excluded by a subsequent denaturation of the renaturation products. Without further addition of dithionite, fully reduced *"phycorubin"* was obtained back. This proves, that reoxidation to the corresponding cyanin had occured under conditions *thermodynamically* favoring the reduced chromophores in *denatured* PC.

The results can, therefore, only be explained by a *thermodynamic* argument, *e. g.* a stability difference of the biliprotein chromophores towards reaction with dithionite between the *native* and the *denatured* state. If the dithionite reaction is a true reduction to rubins (hydrogenation of C-10 and N-22), this would implicate, that the redox potential of the chromophores must be more negativ in the *native* than in the *denatured* state. This stability difference towards reduction may be correlated to the recently suggested conformational change from the cyclic form in the *denatured*, to the extended conformation in the *native* chromoprotein [1]. This possibility is currently investigated by reduction studies on suitable model compounds. A decreased stability towards oxidation has also been found for square planar complexes of bilirubin with metals like Zn^{2+}, and the effect has been correlated to conformational changes [24].

By treatment of native and denatured P_r with dithionite, analogous results were obtained as with the respective forms of PC. *Denatured* P_r was fully reduced, while only partial reduction of *native* P_r was observed at $c_{\text{dithionite}} = 5 \times 10^{-3}$ M. In contrast to PC, however, attempts to renature urea *denatured* phytochrome by gel filtration failed. Though a *kinetic* argument can, therefore, not yet be unequivocally excluded in this case, one may suggest a similar *stability* change of the native P_r chromophore towards reduction by analogy with PC.

An acceleration of the *in vitro* dark reversion of P_{fr} by reductants like dithionite, NADH and ferredoxin has been described by Mumford and Jenner [18]. Under the conditions used in this study, treatment of *native* P_{fr} with 5×10^{-3} M dithionite led to complete conversion to the partially reduced P_r form within 10 min, the time required to take a spectrum. Mumford and Jenner [18] have speculated about some kind of a reduced intermediate during the interconversion. From the experiments described, it may be suggested, that the P_r is produced from P_{fr} via a bilirubin − like species. Kinetic studies are in advance to clarify this proposal.

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