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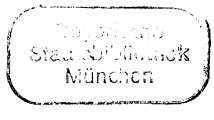

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UV-VIS Absorption Spectra at High Pressure of C-Phycocyanin and Allophycocyanin from *Mastigocladus laminosus*

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Herrn Professor Kurt Schaffner zum 60. Geburtstag gewidmet

Photosynthesis, Phycobiliproteins, Light-Harvesting Pigments, Aggregation Effects, High-Pressure Spectroscopy

The behaviour of monomers and trimers of C-phycocyanin and allophycocyanin under high pressure is studied by UV-VIS absorption spectroscopy. It is found that fast variations of pressure ($\Delta p \geq 500$ bar) and/or temperature are accompanied by significant changes in the absorption spectra (intensity and/or spectral shift). The induced differences disappear, however, in part, when the samples are left for several minutes at the final pressure (relaxation effect). The observed spectral variations are different from those connected with a change in state of aggregation and could therefore be due to small modifications of the chromophore-protein arrangement.

Introduction

Phycobiliproteins constitute the main light-harvesting pigments of cyanobacteria, red and cryptophyte algae [1–4]. Hetero-hexamers (usually termed trimers) of them form the building blocs of the phycobilisomes, extra membraneous antenna particles, which feed their excitation energy predominantly to photosystem II [5, 6]. In the phycobilisomes, several phycobiliproteins are organized in a hierarchical order: α and β subunits constitute the hetero-dimeric protomers of the various individual biliproteins. They can further aggregate to hetero-hexamers (hetero-hexamers) or hetero-dodecamers (hexamers). These basic building blocs are then assembled to the phycobilisome by a complex set of linker peptides, which probably also control the ratio of the individual pigments within the phycobilisome [7, 8]. Crystals of PEC and PC from *Mastigocladus laminosus* are made up of torus-shaped hetero-hexamers, whereas two such units, arranged face-to-face, make up the repeating unit of PC crystals from another cyanobacterium, *Agmenellum quadruplicatum* [9]. In solution, and in the absence of linkers, the aggregate number is somewhat controversial. Hetero-dimers, hetero-hexamers, and/or hetero-dodecamers have been found predominantly in solution of biliproteins from various sources, and the aggregation thermodynamics have been studied in detail in several cases, but hetero-tetramers and other aggregates have been reported as well [2]. It should be noted, however, that most of these studies have been performed before the significance of the linker peptides has been fully appreciated.

The generally applied method of choice to investigate aggregation in biliproteins, is ultracentrifugation ([2], but see [10]). Despite of the fact that this method is fast and the results are well reproducible, it should not be overlooked that the proteins

Abbreviations: APC, allophycocyanin; PC, phycocyanin; PEC, phycoerythrocyanin.

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are exposed to considerable pressures. At 250,000 × *g*, which is common in such experiments, a 1 cm water column above the sample exerts a pressure of 250 bar. Pressures up to several hundred bars are thus experienced by the sample in an analytical cell, and pressures up to and exceeding 1 kbar in a Martin/Ames type analysis [11] using swingout rotors.

Since aggregation of many proteins is known to be affected by pressure of this magnitude [12, 13], pressure effects on biliproteins are of considerable interest, but have, to our knowledge, not been reported before. As aggregation is known to influence the UV-VIS absorption spectra of biliproteins, high pressure absorption spectroscopy is expected to be well-suited to monitor aggregation changes under pressure. In this communication we wish to present data on the behaviour of PC and APC in different states of aggregation under pressures in the range $1 \leq p \leq 1000$ bar.

Experimental

Biliproteins

PC and APC have been isolated from *Mastigocladus laminosus* as reported before [14]. They were prepared in potassium phosphate buffer (100 mM, pH 7) and stored on ice until use a few days later.

In the isolate, APC (A_{650}/cm = 1 cm^{-1}) is present as a hetero-hexamer. Hetero-dimers (A_{620}/cm = 1 cm^{-1}) were prepared by the addition of the chaotrop KSCN [2, 15]. At 1 M KSCN, the pigment is present exclusively as hetero-dimer as determined by ultracentrifugation [11]. The sample referred to as APC-mix (A_{620}/cm = 0.5 cm^{-1}) was obtained by addition of only 0.3 M KSCN.

The aggregation state of PC is concentration dependent. According to ultracentrifugation [11] and the position of the absorption maximum [16], the hetero-hexamer sample (A_{620}/cm = 1 cm^{-1}) was >95% in this state and the PC-mix (A_{620}/cm = 0.5 cm^{-1}) contained a 1:1 mixture with hetero-dimers.

Apparatus

Absorption spectra at ambient pressure were recorded with a Lambda 2 spectrophotometer (Perkin Elmer). The home-made apparatus used for the absorption measurements at high pressure is described in detail by Wokusch [17]. Six samples can be investigated simultaneously in a high pressure reactor (p_{max} = 400 MPa). The high pressure is produced by a motor driven spindle pump; it can be decreased rapidly by a release valve. The six cells are coupled to a diode array spectrophotometer (Model MCS 130, Zeiss, Oberkochen) by fiberoptics and an optical multiplexer and pressure resistent optical probes [18]. Due to the several coupling sites at the multiplexer and the probes, only a fraction of the analyzing light ($\lambda \geq 380$ nm) reaches the detector (50% loss per coupling site). The measuring time for one full spectrum is < 1 sec/sample, and the total sample volume (1.2 ml) is large compared to the volume illuminated during the measurement (0.006 ml). Any influence of the probing beam on the sample, *e.g.* photochemical reactions, should be negligible under these conditions.

The advantage of having more samples under identical conditions (pressure and temperature) is obvious. If one sample cell contains the pure solvent, the effects of pressure and temperature (refractive index change) on the transmission of the probing light beam can be corrected for. Furthermore, the cell construction is such that its diameter is invariant. Since the path of light is parallel to the cell axis, the number of molecules in the lightbeam remains constant during the measurement, and no pressure dependent concentration correction has to be applied (unless the refractive index of the buffer and that of the PC solution change differently under pressure).

To compensate for baseline drifts all spectra were shifted vertically to show zero absorption at 728 nm. No further data manipulations like averaging for noise reduction or normalization were performed and difference spectra are directly calculated from these spectra.

Results

Stability of samples

The samples were chosen with the objective to study well-defined aggregation states. These were for APC: hetero-dimers (obtained by treatment with 1 M KSCN), and hetero-hexamers (see above). The third APC sample with only 0.3 M KSCN contained a mixture of these aggregates (APC-mix). For PC, two samples with different PC concentrations were used to obtain different

aggregation equilibria. The PC hetero-hexamer is >95% pure whereas the more dilute solution represents a ≈ 1:1 hetero-dimer/hetero-hexamer mixture (PC-mix). A concentration yielding hetero-dimer only (A_{620}/cm < 0.1 cm^{-1}) proved unsuitable to obtain spectra with sufficient signal-to-noise ratio. The mixtures were chosen deliberately, because any effect on the aggregation equilibrium is expected to show up most clearly if both species are present in about equal amounts.

All samples were exposed to the pressure – temperature program shown schematically in Fig. 1. First, the pressure was slowly increased by certain amounts and then held constant for short periods of time. Sets of spectra were taken continuously at 1 min intervals during both the increase and after the stop in the pressure increase (until a maximum pressure of 1000 bar was reached). Then, the pressure was rapidly decreased from 1000 to 500 bar, from 500 to 250 and so on. Again the pressure dependence and the relaxation at constant pressure of the absorption spectra were monitored after each step under constant pressure. This first cycle (0 < t < 180 min, corresponding to the time between points A and B) was followed by a second, faster pressure increase – decrease cycle at the same temperature (10 °C). Then, the temperature was increased to 30 °C while the samples were kept under 500 bar pressure (after point c).

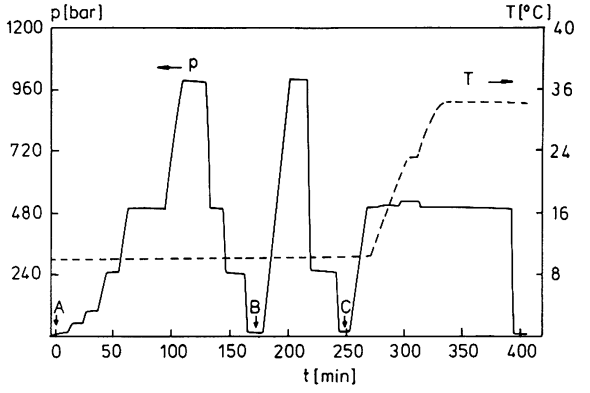

Fig. 1. Pressure (—) and temperature profile (– – –) of the experiment. Each minute a set of spectra (samples 1 through 5 *plus* buffer reference) was recorded. Up to $t = 270$ min, the temperature was kept at 10 °C; it was then raised to 23 and finally to 30 °C. Then, the temperature was constant again for the remaining time. The labels A, B and C correspond to the ones used in the text.

Fig. 2 displays for comparison the spectra of the samples recorded at the beginning of the experiment (point A in Fig. 1) and those taken after each of the pressure cycles (points B and C). They prove the stability of the samples during the pressure treatment. The absorption changes are negligible in the APC hetero-hexamer (Fig. 2c); *e.g.* the decrease in absorption at the maximum is less than 1%. The APC hetero-dimer (Fig. 2e) shows an absorption decrease of about 2% over the entire spectrum. The shape of the difference spectrum corresponds to the absorption spectrum itself, the

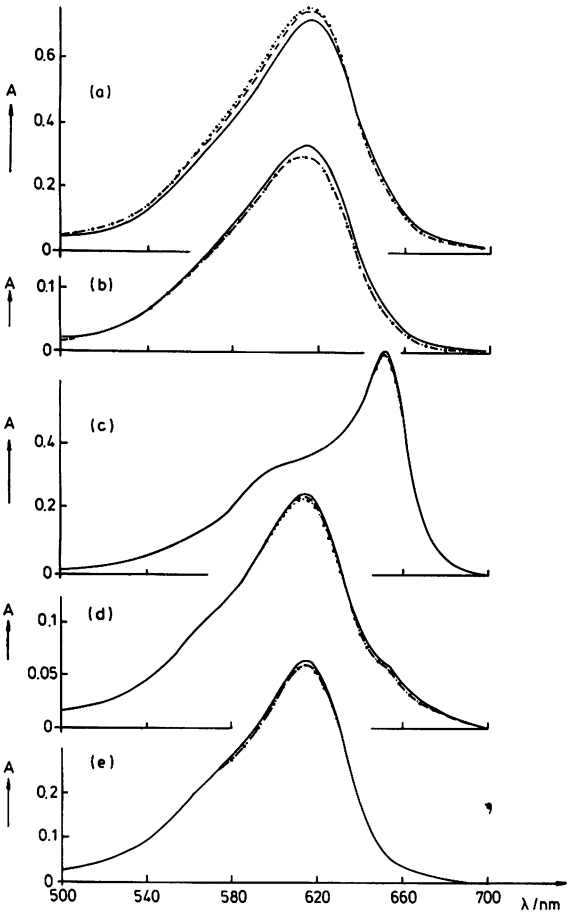

Fig. 2. Absorption spectra at 10 °C and 10 bar at the beginning of the experiment (point A in Fig. 1, —), after the end of the first cycle to 1000 bar and back to 10 bar (point B, – – –), and after the second cycle (point C, ·●·●·). a) PC hetero-hexamer, b) PC mix, c) APC hetero-hexamer, d) APC mix, e) APC hetero-dimer.

decrease could therefore be due to some precipitation. PC mix (Fig. 2b) behaves qualitatively similar to APC hetero-dimer but the effect was considerably larger (6%). Only PC hetero-hexamers (Fig. 2a) show a rather different effect. There is an increase in absorption which amounts to 5% at the maximum, and at the same time a slight blue-shift of the absorption maximum by about 2 nm. Such a band shift is indicative of decreased aggregation. However, dissociation is usually not accompanied by an increase of the molar extinction coefficient, so that some other process must take place in addition. As will be shown below, the major change in the status of this sample took place during the initial rise in pressure, after which the sample remained as stable as the others.

Effect of pressure

The effect of pressure was determined from the spectra (or their differences) taken at the beginning and immediately after the end of the pressure change, that means before the samples had relaxed at the new pressure. In the p-t-profile (Fig. 1) such a pair of points correspond to the endpoints of the plateau before the pressure change, and the first point on the plateau after the pressure change under consideration.

APC hetero-hexamers show a rather complex difference spectrum (Fig. 3c). There is a pronounced absorption increase between 655 and 690 nm and around 615 nm, which is within the limits of error proportional to the pressure increase. In addition, a decrease around 645 nm is observed which is pronounced only above 500 atm. This behaviour does not correspond to a simple change in aggregation, upon which a decrease around 650 and an increase around 620 nm is observed at ambient pressure (see [2]). It appears rather, that a red-shift of the 650 nm component is combined with an overall increase in absorption. The absorption changes recorded upon decreasing and increasing the pressure repetitively indicate a fully reversible spectral change (data not shown). These results demonstrate again the absence of any significant irreversible pressure effect in APC hetero-hexamers.

APC hetero-dimers show a monotonous absorption increase with increasing pressure (≈ 10%/kbar) around 635 nm, that is to the red of the absorption maximum (Fig. 3e). As can be seen from Fig. 2e, the change is completely reversible. The difference spectra resemble somewhat the difference spectra expected for aggregation, except that the maximum increase is around 635 nm and not at 650 nm. More likely is therefore that the origin of the absorption differences is (like in the hetero-hexamers) a general increase in absorption, combined with a red-shift of the maximum.

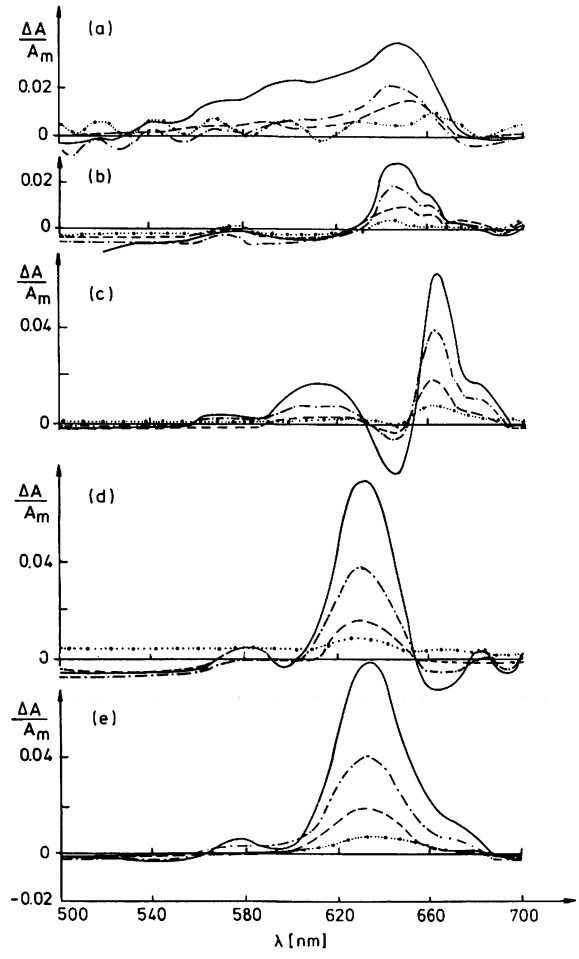

Fig. 3. Effects of increasing pressure on absorption spectra of a) PC hetero-hexamer, b) PC mix, c) APC hetero-hexamer, d) APC mix, e) APC hetero-dimer. Difference spectra at high (values below) *minus* low pressure (10 bar). High-pressure spectra were recorded immediately after reaching the respective setting. (·●·●·) 10 → 100 bar; (– – –) 10 → 250 bar; (–·–·–) 10 → 500 bar; (—) 10 → 1000 bar.

The response of PC hetero-hexamer to the initial pressure increase is small, and the noisy spectra do not give a well-defined trend (Fig. 3a). A similar small change is seen with the PC mix. Upon release of the pressure, the absorption of PC hetero-hexamer shows however a very large decrease (Fig. 4a) which contrasts at first glance with the many times smaller change upon pressure increase (Fig. 3a). Since the effect of increasing the pressure remains small also in the second cycle (see trace ·●·●· in Fig. 4a), the different behavior seems to be related to the different rates at which the pressure changes. Due to experimental limitations, the pressure build-up occurs at a maximum speed of about 50 bar/min, whereas the decrease is as fast as 300 bar/min (Fig. 1). Consequently, any slow relaxation process following the pressure change should show-up much more clearly in that part of the cycle where the pressure changes rapidly.

Indeed, the existence of such a relaxation process is verified by the difference of spectra taken at constant pressure at various times after a large rapid pressure decrease (represented by a plateau in Fig. 1). The shapes of these difference spectra (Fig. 5) are similar to the difference spectra obtained as immediate effect of the rapid pressure decrease, but the sign of the change is opposite. That observation obviously shows that upon fast pressure release there is a significant rapid decrease in absorbance in PC.

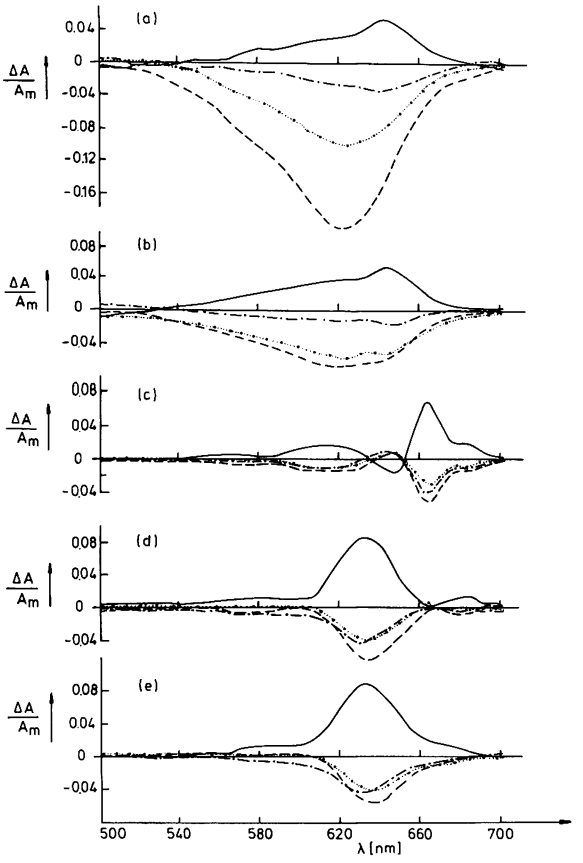

Fig. 4. Effects of decreasing and re-increasing pressure on absorption of PC a) hetero-hexamer, b) PC mix, c) APC hetero-hexamer, d) APC mix, e) APC hetero-dimer. Difference spectra (final *minus* starting) with pressures as indicated below. Starting spectra were measured after relaxation, the final ones immediately after reaching the set pressure. (·●·●·) 1000 → 500 bar; (– – –) 1000 → 250 bar; (–·–·–) 500 → 10 bar; (—) 10 → 1000 bar.

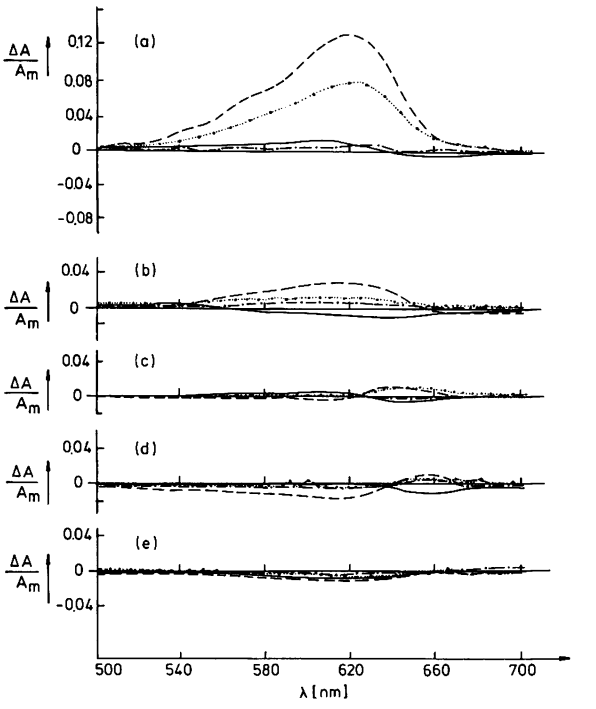

Fig. 5. Transient effects observed in absorption spectra after rapid pressure changes of a) PC hetero-hexamer, b) PC mix, c) APC hetero-hexamer, d) APC mix, e) APC hetero-dimer. The differences (relaxed *minus* unrelaxed) are between spectra which were taken immediately after reaching the final pressure given below and those recorded after some time of relaxation at this pressure. (·●·●·) 1000 → 500 bar; (– – –) 1000 → 250 bar; (–·–·–·) 250 → 10 bar; (—) 10 → 1000 bar.

Yet on a time scale of minutes, the protein-chromophore assembly relaxes into the pressure-adequate equilibrium. During this relaxation process, a part of the rapid absorbance decrease vanishes, such that the long-time net effect of the pressure decrease is rather small and essentially equal in size to the absorbance decrease observed upon a sufficiently slow pressure increase. Therefore, the absorption spectra are restored after passage through the complete pressure cycle (see Fig. 2). The relaxation effect is particularly pronounced for fast pressure changes above 250 bar. That no such relaxation is found after a slow pressure increase (dotted spectrum in Fig. 3) is not surprising in view of the above presented results.

This pronounced relaxation effect is found only in the PC hetero-hexamer. By comparison, the relaxation of APC hetero-hexamer is much smaller, the maximum effect (1000−250 bar) is in the range of 1% (Fig. 5c). A similar small effect is found for the PC-mix sample. It shows the same type of difference spectra as the hetero-hexamer, but with decreased amplitudes. This indicates a negligible effect in the PC hetero-dimer population of the sample. On the other hand, it proves in our opinion that the observed changes in the absorption spectra are not caused by a change in the hetero-dimer/hetero-hexamer ratio as a function of pressure, but rather due to intrinsic effects. In view of the results of X-ray analysis [9], and quantum-mechanical model calculations [19, 20], there are two possible sources to explain the spectral shifts.

According to our present understanding the absorption band profile is inhomogeneously broadened [22], the different contributions originating from various chromophore-protein arrangements. More precisely speaking, there exist different tautomeric forms of the amino-acid residues around the chromophore which, due to electrostatic interactions, give rise to a manifold of chromophore absorption bands. The external pressure change could now cause either a change in free enthalpy of the various chromophore-protein arrangements, thereby changing the statistical weights, with which each conformation contributes, or change the absorption characteristics of some of the statistically important conformations. The latter case seems likely in PC hetero-hexamer, because one knows that there is a strong coupling between the α-84 and β-84 chromophores of neighbouring subunits, which is easily modified by linker peptides. Whichever processes happen in PC hetero-dimer and APC hetero-dimer or hetero-hexamer, they must be much faster or smaller than in PC hetero-hexamer.

Temperature effects

Since at present the origin of the absorption relaxation is unclear, one could principally argue that the rapid pressure decrease could lead to a temperature change, and temperature is known to have a pronounced effect on the absorption of PC from *Spirulina platensis* [21]. However, the apparatus is thermostated to within ±0.1 °C and the *in situ* temperature measurement does not indicate any fluctuation larger than ±0.1 °C (see Fig. 1). Moreover, there is a similar temperature effect expected for APC-samples, which has not been observed. Furthermore, in all samples the difference spectra upon pressure release were similar in shape and magnitude at 10 and 30 °C (not shown).

In a second set of experiments, the temperature was slowly increased from 10 to 30 °C at a pressure of 500 bar (this pressure was chosen, because the relaxation effect of PC was very small below 250 bar). The 20 °C temperature difference is considerably higher than any possible temperature variation in the samples during the previous experiments. With PC hetero-hexamer, there is an absorption decrease by 10% peaking around 623 nm, when measured immediately after reaching the final temperature (Fig. 6a, − − −). This is partly reverted by a subsequent increase if the sample is kept under constant conditions (−•−•−), *i.e.*, there is a relaxation as well as a permanent effect. The remaining effect is complex, as indicated by the extrema in the difference spectrum for the relaxed samples (− − −). The absorption relaxation occurs again on a time scale of several minutes. The temperature induced changes in the PC-mix are also qualitatively different from the hetero-hexamer, in particular there is no second negative band at ≈655 nm.

None of the difference spectra between relaxed samples corresponds to known difference spectra obtained for changes of aggregation state. Although contributions from the latter cannot be excluded, they seem to play a minor role as concluded from a comparison of the results for heterohex-

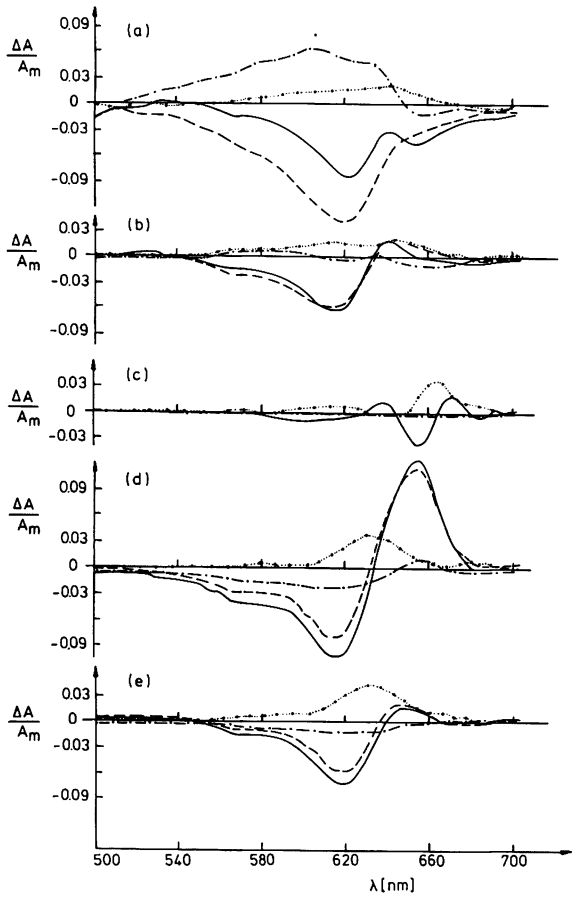

Fig. 6. Effects of increasing temperature at 500 bar with a) PC hetero-hexamer, b) PC mix, c) APC hetero-hexamer, d) APC mix, e) APC hetero-dimer. (·●·●·): Difference spectra induced by a pressure increase from 10 to 500 bar at 10 °C (final *minus* start, no relaxation). (– – –): Difference spectra induced by temperature increase (10 to 30 °C, no relaxation) at 500 bar. (–·–·–): Difference spectra of the relaxation process after temperature increase from 10 to 30 °C at 500 bar (relaxed *minus* unrelaxed). (—): Persistent temperature effect on absorption, difference spectrum (30 °C, relaxed *minus* 10 °C, relaxed). In part 6c, the solid and the dashed traces are identical within the limits of error.

americ pigments, and those samples, which contain hetero-dimers and hetero-hexamers in equilibrium. The effect is always more pronounced in the PC hetero-hexamers which are away from equimolar equilibrium. The opposite would be expected, if the pressure and temperature effects were due to a change in aggregation. The data show at the same time that the hetero-hexamers are much more susceptible to p/T changes, than are the hetero-dimers, which is most likely related to a change in the interaction between α-84 and β-84 chromophores on adjacent hetero-dimers in the hetero-hexamer.

We conclude from this, that i) both the PC hetero-hexamer and the hetero-dimer spectra are temperature-dependent, ii) that the changes are different in these two states, and iii) that no conclusions can be presently drawn concerning any aggregation changes in the process.

In the case of APC hetero-hexamer, the temperature induced difference spectrum is similar to the pressure induced one, but of reversed sign, *e.g.* the 660 nm absorption difference is positive for pressure increase (Fig. 6), and negative for a temperature increase. Again this effect shows only little relaxation with time when the sample is kept under these conditions. The changes in the (partially) dissociated APC are different from the ones in the hetero-hexamer. In this case the highest amplitude is observed in the "APC-mix", indicating that it is (at least in part) related to a shift in the aggregation equilibrium. The s-shaped difference signal with extrema near the absorption maxima of the hetero-hexamer and hetero-dimer, respectively, supports this view. However, the relative amplitudes are different from a simple disaggregation as observed *e.g.* by addition of KSCN [15]. Again, the temperature-induced changes are qualitatively different from the pressure-induced ones, confirming that the latter are unrelated to any heating/cooling effect inadvertently induced by the pressure changes.

Conclusion

One of the important technological aspects of this work is the fact that the biliproteins under study exhibit an immediate change in absorption spectra upon fast pressure or temperature changes. Since this initial change is followed by a relaxation phenomenon which occurs on the time scale of several minutes, the initial drastic changes can be disguised, if the whole spectrum is not recorded within a comparably short time. It is only because the present apparatus applies a diode array spectrometer in combination with optical multiplexing technique and fast data transfer and storage that the evolution of the spectral features can be fol-

lowed with, at least in the PC-samples, sufficient speed and accuracy (S/N-ratio). One must suspect that a fast response to pressure and temperature changes occurs in many more chromoproteins, than has been reported so far, just because the recording by the spectrometers was not sufficiently fast. *E.g.* the change in absorption of PC heterohexamers is significantly different when measured under quasistationary conditions than under pressure or temperature jump. The results further indicate that the effect of temperature and pressure jump are similar in spectral shape, but opposite in sign. APC and PC differ as such as the changes in PC seem to be transient in nature, that is they compensate when a temperature or pressure cycle is completed. In contrast, APC shows less or no relaxation after change of p or T, and some of the induced changes are present after completion of the cycle indicating persistent changes in the sample induced by higher pressure or/and temperature.

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