Reconstitution of an Allophycocyanin Trimer Complex Containing the C-Terminal 21–23 kDa Domain of the Core-Membrane Linker Polypeptide L_{cm}

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Allophycocyanin (AP) was isolated from extracts of the cyanobacterium *Mastigocladus laminosus*. A fraction enriched in AP-associated polypeptides with apparent molecular masses of 21–23 kDa in SDS-PAGE, was isolated on a preparative scale and identified as a homologous mixture of C-terminal fragments of the core-membrane linker polypeptide L_{cm}. The complex $(\alpha^{AP}\beta^{AP})_3 \cdot 21-23$ kDa was reconstituted and characterized by sucrose density gradient ultracentrifugation, absorption, fluorescence emission and circular dichroism spectroscopy. The 21–23 kDa polypeptides were found to induce spectral changes in AP similar to those induced by the small core linker polypeptide $L_c^{8.9}$. Possible functions of the complex in phycobilisomes are discussed.

Introduction

Phycobilisomes, the light-harvesting antennae of cyanobacteria, red algae and cyanelles, are supramolecular, highly-structured protein complexes located on the thylakoid surface (Wehrmeyer, 1983; Gantt, 1986). They are composed of chromophore-bearing phycobiliproteins (Scheer, 1982; Glazer, 1985; MacColl and Guard-Friar, 1987) absorbing light over a wide spectral range, and of linker polypeptides which regulate and stabilize the phycobilisome structure, but also modify the light-absorption properties of the phycobiliproteins (Tandeau de Marsac and Cohen-Bazire, 1977; Bryant, 1991).

Abbreviations: PC, phycocyanin; PEC, phycoerythrocyanin; AP, allophycocyanin; APB, allophycocyanin B; α, β, biliprotein subunits; L, linker polypeptide, subscripts denoting origin and function in phycobilisomes (r, rod; c, core; rc, rod core; cm, core membrane), superscripts denoting molecular weight in kDa; OD, optical density; OD · ml, unit of protein quantity, OD multiplied by sample volume in ml, subscripts denoting wavelength in nm; SDS-PAGE, sodium dodecylsulfate-polyacrylamide-gel-electrophoresis; CD, circular dichroism; p/s, peak (λ_{max})-to-shoulder (600 nm) absorbance ratio; $\Delta A/A_{max}$, ratio of CD signal intensity to absorbance value at λ_{max}.

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In hemidiscoidal phycobilisomes, an array of rods is linked by one or more rod-core linker polypeptides to the phycobilisome core. The bi- or tricylindrical core substructures are formed by allophycocyanin (AP), two minor biliproteins structurally related to AP, and the two linkers L_c and L_{cm}. The latter, a large polypeptide of 127 kDa molecular mass in the cyanobacterium *Mastigocladus laminosus* (Esteban, 1993), contains several internal repeats and plays a singular role. It anchors the core to the membrane (Redlinger and Gantt, 1982; Rusckowski and Zilinskas, 1982; Reuter and Wehrmeyer, 1990), and is involved in the organization of the core, but also carries a chromophore which is probably the terminal emitter to photosystem II (Gindt *et al.*, 1992; Zhao *et al.*, 1992).

The key role of the linker polypeptides in the aggregate formation and the fine-tuning of the absorption characteristics of the phycobiliproteins has been widely studied, but is still only poorly understood. A technique for the isolation of linker polypeptides and their fragments and for the reconstitution to biliprotein-linker complexes recently developed in our laboratory (Gottschalk *et al.*, 1991 and 1993), has facilitated the study of these proteins. Here, we present results on a complex of AP-trimer with C-terminal fragments of the L_{cm} polypeptide, which shows a strong hyper-

chromism as compared to AP-trimers without linker. Our findings may be significant in the present discussion of the phycobilisome architecture of this organism.

Materials and Methods

Absorption and CD measurements were made at an optical density of 0.5–0.75. Fluorescence emission was measured at an optical density of 0.1 at the absorption maximum in 1 cm cells; excitation was at 580 nm. *Allophycocyanin* was isolated from *Mastigocladus laminosus* on DE 52 (Whatman), pre-purified on hydroxylapatite Bio-Gel HT (Bio-Rad), and purified on Fractogel TSK DEAE-650(2) (Merck) from all contaminations of other biliproteins and of linker polypeptides by the methods previously described (Gottschalk *et al.*, 1991 and 1993; Gottschalk, 1993). *21–23 kDa polypeptides* were isolated with buffers containing 3.5 M urea (Serva, p.a. grade) from pre-purified AP on S-Sepharose Fast Flow (Pharmacia) as described previously for $L_c^{8.9}$ (Gottschalk *et al.*, 1993). *Reconstitution experiments* – 6 OD_{620}·ml AP (in 230 µl 30 mM potassium phosphate buffer, pH 7, containing 3.5 M urea) and 1.2 OD_{276}·ml enriched fraction of 21–23 kDa polypeptides (in 400 µl 10 mM Tris/HCl, pH 8.9, containing 3.5 M urea and 100 mM KCl) were mixed, reconstituted in potassium phosphate buffer (700 mM, pH 7.5), purified by sucrose density gradient ultracentrifugation and characterized by methods described previously for complexes containing $L_c^{8.9}$ (Gottschalk *et al.*, 1993). The *aggregation state* was determined by 3–12% sucrose density gradient ultracentrifugation in 700 mM potassium phosphate buffer (pH 7.5) as described previously (Gottschalk *et al.*, 1993). *Amino acid sequences* – LysC digestion of protein bands in polyacrylamide matrix was followed by elution and HPLC separation of the fragments (Eckerskorn and Lottspeich, 1989). N-terminal sequences of the fragments were determined as described previously (Eckerskorn *et al.*, 1988; Lottspeich, 1985). *SDS-PAGE* was done according to Lämmli (1970), using a 5% stacking gel and a 15% separation gel.

Results

In a previous publication, a new method for the isolation and purification of biliproteins and linker polypeptides and for reconstitution of biliprotein-linker complexes has been introduced and applied to the reconstitution of $(\alpha^{AP}\beta^{AP})_3$ and of $(\alpha^{AP}\beta^{AP})_3 \cdot L_c^{8.9}$ (Gottschalk *et al.*, 1993). During this work, another reconstituted complex, $(\alpha^{AP}\beta^{AP})_3 \cdot 21-23$ kDa, had been prepared (Gottschalk *et al.*, 1993), but could not be further characterized then because the three 21–23 kDa polypeptides were N-terminally blocked. The related polypeptides were now enriched by chromatography on S-Sepharose FF. The fraction of 21–23 kDa polypeptides eluting at about 100 mM KCl was still contaminated by other polypeptides (Fig. 1, lane 1). However, the major three bands at apparent molecular masses of 21–23 kDa were the only ones in this fraction reconstituting stable complexes with AP (Fig. 1, lane 3), whereas the contaminations precipitated and were lost during the reconstitution and subsequent purification procedure. Therefore, under circumstances favouring specific interactions, reconstitution can be used to obtain pure and specific complexes from impure samples. The polypeptides of the three bands forming a complex with AP were separately cleaved with LysC, and some of the fragments were sequenced (Table I). All bands contain cleavage products identified as fragments of the C-terminal part of L_{cm}, by comparison with the

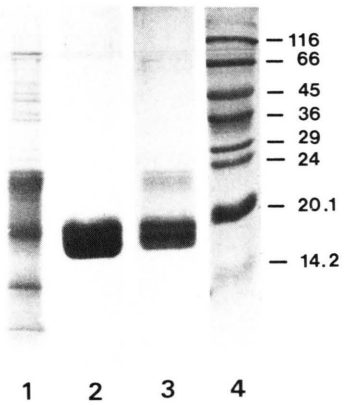

Fig. 1. SDS-PAGE of isolated polypeptides and reconstituted complexes. Lane 1: fraction of 21–23 kDa polypeptides isolated from S-Sepharose FF column. Lane 2: $(\alpha^{AP}\beta^{AP})_3$ complex reconstituted as a control. Lane 3: reconstituted $(\alpha^{AP}\beta^{AP})_3 \cdot 21-23$ kDa complex. Lane 4: molecular weight markers (14.2, 20.1, 24, 29, 36, 45, 66 and 116 kDa).

Table I. Sequences of LysC fragments of the upper, middle and lower band of 21–23 kDa polypeptides in the reconstituted complex with AP (Fig. 1, lane 3), and amino acid positions of identical sequences of L_{cm} from *Mastigocladus laminosus* (Esteban, 1993).

Band	Sequences	Position
upper	LLADGGLK	1063–1070
	AAADLVK	1110–1116
middle	FYTPYPNTK	1029–1037
	LLADGGLK	1063–1070
lower	FYTPYPNTK	1029–1037
	LLADGGLK	1063–1070
	AAVEAMVNSP	1071–1080

amino acid sequence deduced from the DNA sequence (Esteban, 1993).

Esteban (1993) has recently also identified a 23 kDa polypeptide as a C-terminal fragment of L_{cm} starting at amino acid position 923. The similar size and sequence overlaps show that our polypeptides are closely related to the latter. Since they are N-terminally blocked, it could not be determined whether they differ from each other N- or C-terminally. The finding of this family of closely related L_{cm} fragments can be rationalized by an attack of an endogenous protease or *M. laminosus* on regions of L_{cm} not protected inside an AP trimer after subjecting phycobilisomes to dissociating conditions. 21–23 kDa seems to be a common size for stable fragments of linker polypeptides protected inside biliprotein trimers against further degradation (Yu and Glazer, 1982; Lundell *et al.*, 1981; Lundell and Glazer, 1983a; Gottschalk *et al.*, 1991; Glauser *et al.*, 1992b); we suggest that the C-terminal domain of L_{cm} is following the same principle.

The basis for the N-terminal block of our isolates is unclear. N-terminally blocked linker polypeptides have been reported from other organisms (Lundell *et al.*, 1981; Glauser, 1991; Glauser *et al.*, 1992b), but are not expected for proteolytic fragments. A block could be due to reaction with cyanate ions in the 3.5 M urea solution used throughout the isolation, although no such effect has been observed previously in our work with biliproteins and linker polypeptides of *M. laminosus* isolated under similar conditions (Gottschalk *et al.*, 1991 and 1993; Gottschalk, 1993).

Complete complexation of trimeric AP with the 21–23 kDa polypeptides was ensured by the use of an excess of the latter, as discussed in Gottschalk *et al.* (1993). The reconstituted $(\alpha^{AP}\beta^{AP})_3 \cdot 21$–23 kDa complex was predominantly ($\geq$ 85%) trimeric; only traces of monomeric ($\leq$ 8%) and hexameric ($\leq$ 7%) aggregates could be detected by sucrose density gradient ultracentrifugation. Only the trimeric fraction from the gradient was used for the spectra shown in Fig. 2. No contamination by APB or the chromophore-bearing N-terminus of L_{cm} was detected by spectroscopic methods or SDS-PAGE.

The red absorption band of the reconstituted complex was at λ_{max} = 651.5 nm, and had an extremely high $^{P}/_{S}$-ratio of up to 2.95; the fluorescence emission was at λ_{max} = 663 nm (Fig. 2a). The complex gave very strong CD signals with $\Delta A/A_{max} = 4.7 \cdot 10^{-4}$ (Fig. 2). When compared to the spectra of reconstituted $(\alpha^{AP}\beta^{AP})_3$ and $(\alpha^{AP}\beta^{AP})_3 \cdot L_c^{8.9}$ (Gottschalk *et al.*, 1993), the absorption and fluorescence emission maxima of the new complex are at intermediate values.

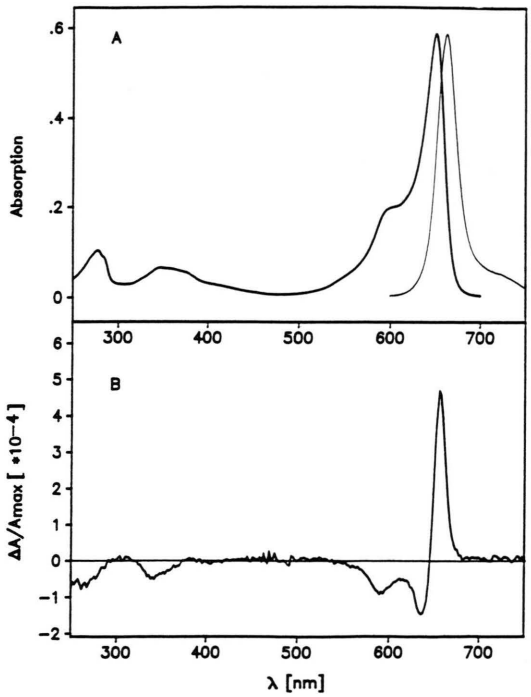

Fig. 2. Absorption (**A**, heavy line, left ordinate), fluorescence emission (**A**, thin line, right ordinate) and CD spectra (**B**) of the reconstituted $(\alpha^{AP}\beta^{AP})_3 \cdot 21$–23 kDa complex.

However, $^{p}/_{s}$ = 2.95 is the highest value measured for any AP-complex as yet. The overall shape and the signal positions of the CD spectrum are similar to $(\alpha^{AP}\beta^{AP})_3 \cdot L_c^{8.9}$, but the signal intensities are rather different. $(\alpha^{AP}\beta^{AP})_3 \cdot 21-23$ kDa shows the highest value of $\Delta A/A_{max}$ for the three complexes, while the other linker-containing complex, $(\alpha^{AP}\beta^{AP})_3 \cdot L_c^{8.9}$, gives the lowest amplitudes ($\Delta A/A_{max} = 1.5 \cdot 10^{-4}$; Gottschalk *et al.*, 1993). In $(\alpha^{AP}\beta^{AP})_3 \cdot 21-23$ kDa, the negative signal at around 635 nm is more pronounced than that at around 594 nm; in $(\alpha^{AP}\beta^{AP})_3 \cdot L_c^{8.9}$, the intensities of these signals are reversed.

Discussion

L_{cm} is the largest and most complex linker in phycobilisomes. It plays a central role in the energy transfer from the rods through the core to the photosystems. Specific interactions with the biliproteins are necessary to explain the functions of L_{cm} as a structural protein of the core, as the anchor protein to the thylakoid membrane, as a linker modifying the spectral properties of the associated biliproteins, and as the presumed terminal emitter of the phycobilisome (Redlinger and Gantt, 1982; Rusckowski and Zilinskas, 1982; Lundell and Glazer, 1983a/b/c; Houmard *et al.*, 1990; Capuano *et al.*, 1991; Gindt *et al.*, 1992; Zhao *et al.*, 1992). Complexes containing L_{cm} have been isolated only as large core fragments containing also other linkers and biliproteins (Lundell and Glazer, 1983a/c; Isono and Katoh, 1987; Reuter and Wehrmeyer, 1990). AP-complexes containing a single defined linker have been described only for $L_c^{8.9}$ and rod-core linkers (Lundell and Glazer, 1983b; Füglistaller *et al.*, 1987; Glauser *et al.*, 1990; Gottschalk *et al.*, 1993).

We have shown here, that the C-terminal domain of L_{cm} is capable of forming specific complexes with AP trimers. This domain is not singular in L_{cm}. Following a N-terminal biliprotein domain, L_{cm} polypeptides contain two to four such repeats, which are also homologous to the N-terminal domains of rod and rod-core linkers (Bryant, 1988; Houmard *et al.*, 1990). In phycobilisomes containing L_{cm} with four repeats, the C-terminal domain has been suggested to link PC (Capuano *et al.*, 1991) or AP-hexamers (Isono and Katoh, 1987; Bryant, 1991) at the basis of two of the rods to the core. This would define the C-terminus of the L_{cm} of *M. laminosus* and *Anabaena sp.* PCC7120 as a rod-core linker, and would have to be taken into account with regard to the proposed stoichiometries of these polypeptides (Glauser *et al.*, 1992a). Rods with AP at their base would either require the interaction of $L_r^{34.5, PC}$ with AP, binding the outer PC-hexamers to the basal AP-disc. Alternatively, one of the presumed rod-core linkers would have to play this role, being in reality a misnamed rod linker. In this context it is interesting that an $(\alpha\beta)_6^{PC} \cdot L_{rc}^{29.5} \cdot (\alpha\beta)_3^{AP} \cdot L_c^{8.9}$ complex of unknown morphology and location within the phycobilisome has been isolated and reconstituted by Glauser *et al.* (1993). This so-called rod-core complex could then also be part of a special rod type.

It should be pointed out, however, that the experimental evidence supporting this specific phycobilisome model (Bryant *et al.*, 1991; Glauser *et al.*, 1992a/b; Esteban, 1993) is still ambiguous. Our results are also compatible with other models: the complex containing the C-terminal domain of L_{cm} could be involved in the interaction between neighbouring phycobilisomes, and it could also be an integral part of the core.

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