CHLOROPLAST AND NON-CHLOROPLAST ADENOSINE-3′:5′-CYCLIC-MONOPHOSPHATE-RECEPTOR-PROTEINS IN *EUGLENA GRACILIS*

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1. Introduction

In mammalian cells and in *Escherichia coli*, cyclic AMP and its receptor proteins acts as intermediates in the control of numerous cellular activities. In photosynthetic organism, cyclic AMP has been found in *Chlamydomonas reinhardtii* [1] and *Euglena gracilis* [2,3], whereas results on higher plants are still contested [4–6]. Little is known about the nature of cellular receptors of cAMP in photosynthetic organisms. Keirns et al. [3] described a protein-kinase in *Euglena gracilis* which is slightly stimulated by cAMP. In previous work we have shown the presence of soluble proteins extracted from *Euglena* cells, able to bind cAMP [2].

We demonstrate here that *Euglena* chloroplasts extracted from green cells contain a soluble protein which binds cAMP. This protein is different than that isolated from dark grown cells.

2. Materials and methods

Euglena gracilis strain Z (1224–5/25) was used. Cells were grown in the dark at 25°C in erlenmeyer flasks containing NCb medium [7]; cell density was approx. 5.5×10^6 cells/ml. For chloroplast isolation, green cells were grown in 20-liter carboys containing 15 l of NCb medium with the B_{12} concentration reduced to 0.15 μg/l. Intact chloroplasts were extracted according to the technique described by Schwartzbach et al. [8]; preparations were free of whole cells and cellular fragment contamination was low.

Extraction was performed at 0–4°C following the flow chart shown in fig.1.

Binding of cAMP was measured according to the methods used for cAMP assay involving binding protein [9,10]: the binding reaction was conducted in a vol. of 200 μl in 52.5 mM KH_2PO_4 buffer, pH 6.2, and incubated for longer than 60 min at 0°C. The other components of the incubation mixture were 10 mM $MgCl_2$, [^{3}H]cAMP in variable concentration, unlabeled AMP at a concentration of 10 000 times that of [^{3}H]cAMP, and chloroplast or dark grown cell extract (100 μg protein). The suspension was then filtered either directly or after dilution with 4 ml of cold buffer III (50 mM KH_2PO_4, pH 6.0) through 25 mm millipore filters (0.45 μm) previously rinsed with buffer III. Filters were washed with 12 ml of this buffer, dried and dissolved in 1 ml of methyl cellosolve (ethylene glycol monomethyl ether) in counting vials. Each vial received 10 ml of a scintillation mixture (2 liters toluene; 1 liter Triton X-100; 16.5 g PPO; 200 mg POPOP); radioactivity was measured with a Packard 3375 Scintillation Spectrometer. For each binding condition used, a sample was prepared without cell extract; the background so determined was substracted from the value obtained in presence of the extract.

Protein concentration was estimated by the

Abbreviations: cAMP: adenosine-3′:5′-monophosphate, cyclic; cGMP: guanosine-3′:5′-monophosphate, cyclic; cIMP: inosine-3′:5′-monophosphate, cyclic; b-cIMP: 2′-O-butyryl-inosine-3′:5′-monophosphate, cyclic; db-cAMP: N^6-2′-O-dibutyryl-adenosine-3′:5′-monophosphate cyclic; AMP: adenosine-5′-monophosphate; CRP: cyclic AMP-Receptor-Protein.

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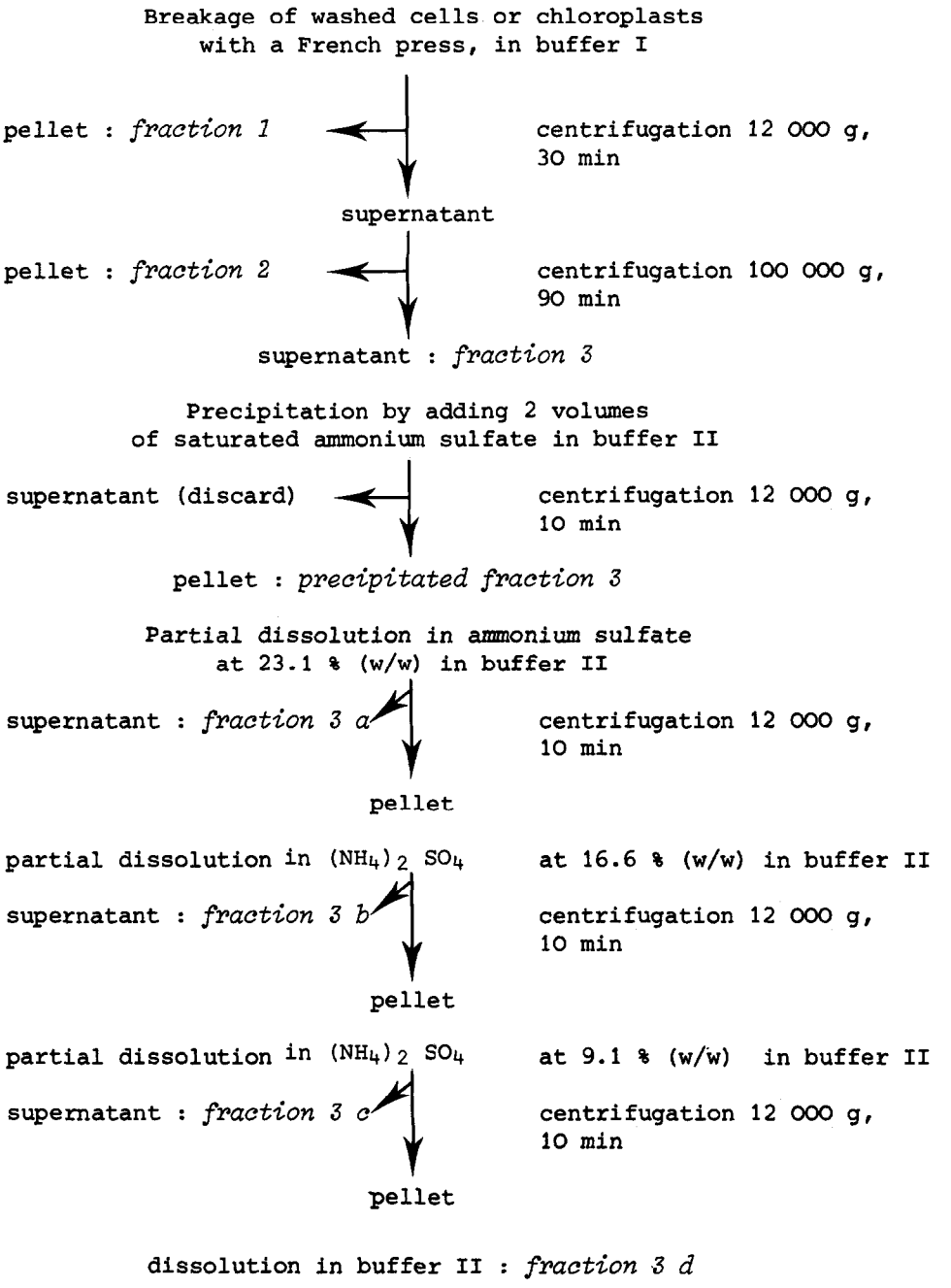

Fig.1. Preparation of extracts from dark-grown cells or chloroplasts. Buffer I: Tris 20 mM, $MgCl_2$ 10 mM, dithiothreitol 1 mM, HCl, pH 7.5. Buffer II: KH_2PO_4 5 mM, dithiothreitol 1 mM, KOH, pH 6.8. Some steps of the extraction procedure may be omitted: when the 30 min centrifugation at 12 000 *g* was omitted the pellet obtained after the 100 000 *g* centrifugation was called *'fraction 1 + 2'*. When the *'precipitated fraction 3'* was directly resuspended into 16.6% (w/w) ammonium sulfate the supernatant obtained after centrifugation was called *fraction 3(a + b)*, and the pellet dissolved in buffer II was called *fraction 3(c + d)*. All the fractions obtained were dialysed overnight against buffer II, then stored at −30°C after freezing into liquid nitrogen.

method of Lowry et al. [11] with bovine serum albumin as standard.

Cyclic [^{3}H]AMP obtained from CEA (France) had a specific activity of 27 Ci/mmol; the radiochemical purity was periodically controlled by high-voltage electrophoresis according to the technique of Verbert and Cacan [12]. Nucleotides were purchased from Boehringer Mannheim except for cIMP which was obtained from Sigma.

3. Results and discussion

Cyclic AMP binding is observed in all fractions from dark grown cells extract, whereas it is only detected in the soluble fraction from isolated chloroplasts (table 1). Disappearance of binding after pronase treatment shows that this property is related to a protein structure.

Total binding of labeled cAMP, measured without AMP, is composed of AMP-sensitive binding and AMP non sensitive binding. Similar results have been described with *E. coli* CRP [13]. AMP-sensitive binding is abolished by the presence of AMP (table 1 and fig.2). AMP-non sensitive binding, which remains in the presence of a large excess of AMP, is lowered in the presence of unlabeled cAMP (fig. 2).

The AMP-sensitive binding is rapidly reduced by dilution, whereas AMP-non sensitive binding is not; so filtration of the incubation mixture was done without dilution (see Materials and methods) when a measurement of an AMP-sensitive binding was included in the experiment. However, more reproducible results were obtained with dilution before filtration.

The AMP-sensitive binding factor is partly separated from the AMP-non sensitive binding protein by

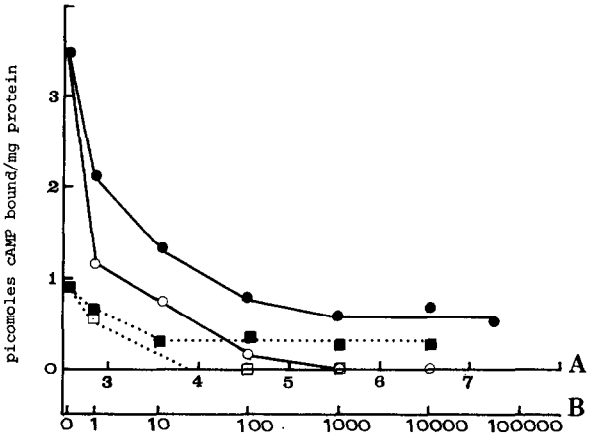

Fig.2. AMP and cAMP competition. Abscissa: A: log concentration (in nM) of (labeled cAMP + unlabeled nucleotide); B: $\frac{\text{unlabeled nucleotide concentration}}{\text{labeled cAMP concentration}}$. Binding with *fractions 3(c + d)* from dark-grown cells (——) or from chloroplasts (.....) in presence of unlabeled 5'-AMP (●, ■) or unlabeled cyclic AMP (○, □). Conditions: [^{3}H]cAMP: 355 nM; filtration without dilution.

Table 1
Cyclic AMP binding with different cellular fractions

Fractions	Bound cAMP (pmoles/mg proteins)			
	Fractions from dark-grown cells		Fractions from chloroplasts	
	without AMP	with AMP	without AMP	with AMP
1	0.827	0.298		
1 + 2			0	0
2	1.360	0.281		
3(a + b)	18.354	0.264	0.264	0.032
3(c + d)	4.980	0.888	0.585	0.378

Conditions: [^{3}H]cAMP: 390 nM; AMP (when present) 3700 μM; filtration without dilution.

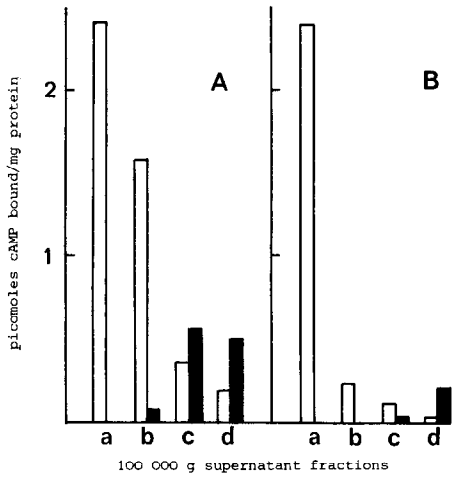

Fig.3. Fractionation of 100 000 *g* supernatant by ammonium-sulfate. A: dark-grown cells; B: chloroplasts. The fractions are called as explained in fig.1. ▭ AMP-sensitive binding; ▬ AMP-non sensitive binding. Conditions: [^{3}H]cAMP: 83 nM; 5′-AMP (when present): 500 μM; filtration without dilution.

fractionating the 100 000 *g* supernatant with ammonium sulfate (fig.3).

With *fractions 3(c + d)* prepared from chloroplasts or dark-grown cells, AMP-non sensitive binding is hardly modified by the incubation mixture at pH 5 to 8.

In order to test the specificity of AMP-non sensitive binding, different unlabeled nucleotides were added to the incubation mixtures and their ability to compete with [^{3}H]cAMP for binding sites was determined (table 2). The results indicate that the binding proteins from dark-grown cells and chloroplasts are relatively specific for cAMP; ATP, ADP and AMP do not interfere. Similar competitions have been described with *E. coli* CRP [14] or the regulatory subunit of a protein-kinase [10].

Determination of the apparent binding constants (K_D) using *fractions 3(c + d)* shows that the binding-protein from dark-grown cells is different from the chloroplast one (fig.4): cAMP binding by extracts from dark-grown cells shows an allosteric pattern, with a constant value of 2.5×10^{-8} M; cAMP binding by chloroplast extracts is not allosteric and shows a constant value of 7.0×10^{-9} M. Only one binding protein is detected in each of the tested fractions as can be seen using a wide range of [^{3}H]-cAMP concentration.

In binding experiments without AMP, when the dark-grown cells *fraction 3 a* which contains only the AMP-sensitive binding (cf. fig.3) is used, one K_D is obtained around 2×10^{-7} M; with the dark-grown cells *fraction 3* two values of K_D are determined: the 2.5×10^{-8} M value, typical of the AMP-non sensitive binding, and the $2–3 \times 10^{-7}$ M value, typical of the AMP-sensitive binding.

Table 2
Competition by unlabeled nucleotides
for the AMP-non sensitive cAMP-binding

Nucleotide	Fraction 3(c + d) from dark-grown cells	Fraction 3(c + d) from chloroplasts
cAMP	93	80
cIMP	79	48
cGMP	35	40
b-cIMP	18	12
db-cAMP	16	0

Results are expressed as:

$$100 \times \frac{\text{(binding without unlabeled nucleotide)} - \text{(binding with unlabeled nucleotide)}}{\text{binding without unlabeled nucleotide}}$$

Conditions: [^{3}H]cAMP: 440 nM; AMP: 370 μM; unlabeled nucleotides (when present): 18.5 μM; dilution before filtration.

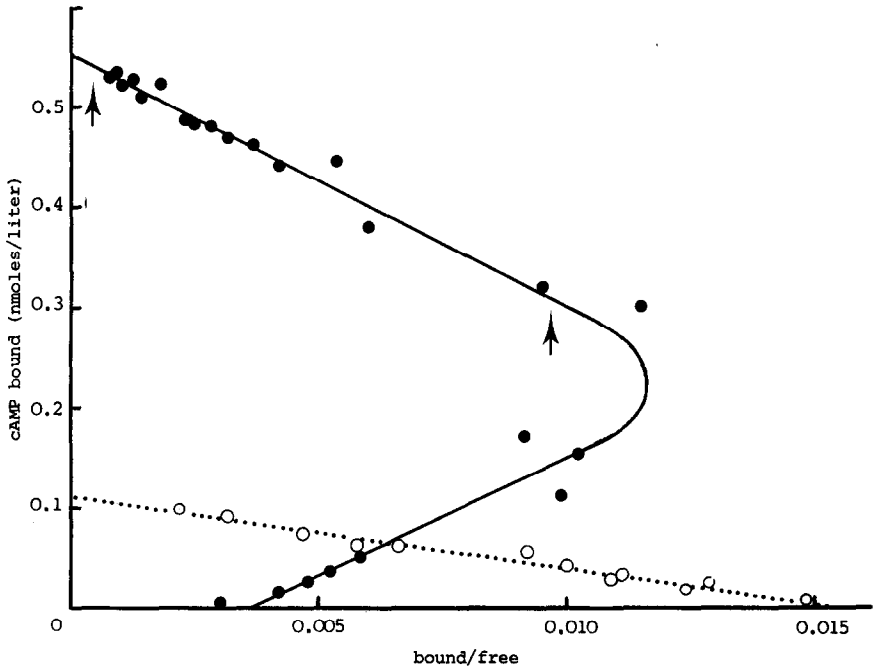

Fig.4. Scatchard plots for the determination of the apparent binding constants. (•–•–•) *fraction 3(c + d)* from dark-grown cells; (○··○··○) *fraction 3(c + d)* from chloroplasts. Conditions: [^{3}H]cAMP: range of concentration from 0.5 to 650 nM; AMP: concentration 10 000 times that of [^{3}H]cAMP; filtration after dilution. K_D determination: the slope of the linear part of the curve equals the negative of the binding constant. The arrows indicate the limits of the part of the curve used to determine the slope with the dark-grown extract.

This study shows the presence of a soluble chloroplast cAMP binding protein in *E. gracilis*. Its binding characteristics are different from those of dark-grown cell extracts. Dark-grown cells and chloroplasts contain at least one protein which binds cAMP non specifically.

The function of these proteins remain unknown. It is not impossible that one of the AMP-sensitive or AMP-non sensitive binding-proteins is a cAMP-phosphodiesterase (EC 3.1.4.17).

Other hypotheses can be considered for the AMP-non sensitive cAMP-binding proteins: one or both may be either a regulatory subunit of a protein-kinase (EC 2.7.1.37) [15] or a CRP which could act as the protein described in *E. coli*. Keirns et al. [3] describe a slightly stimulated protein-kinase in the 30 000 *g* pellet from green cells; this is in keeping with our finding of a cAMP binding factor in the 12 000 *g* and the 100 000 *g* pellet; but the AMP-non sensitive binding proteins we describe are in the 100 000 *g* supernatant.

Wellburn et al. [16] report that the cAMP content in isolated intact *Avena sativa* chloroplasts increases during illumination. We observe large changes in the cAMP content of *Euglena gracilis* after illumination of dark-grown cells [2]. Exogenous cAMP stimulates RNA synthesis in isolated *E. gracilis* chloroplasts [17]. These facts and the results presented here indicate that cAMP may have a regulatory function in the development of proplasts (or etioplasts) to chloroplasts.

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