# Analysis of nucleotides bound to the ATP synthetase from spinach chloroplasts isolated under different conditions[1]

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*Summary.* The total amount of bound adenine nucleotides in the coupling factor isolated from spinach chloroplasts and its distribution on AMP, ADP and ATP was analyzed after various incubation conditions. During purification of the coupling factor, the distribution of AMP, ADP and ATP is not altered. The coupling factor from deenergized membranes contains approximately 1 ADP, less than 1 ATP, and small amounts of AMP. During phosphorylation the pattern is changed and ATP becomes the dominant species. When exogenous ADP is lacking, phosphate is readily incorporated into ATP. Inhibition of adenylate kinase by $AP_5A$ does not change the distribution pattern of the adenine nucleotides. The distribution pattern shows no integer numbers for the different nucleotides, suggesting that the coupling factor is present in different states in a statistical distribution.

## *Introduction*

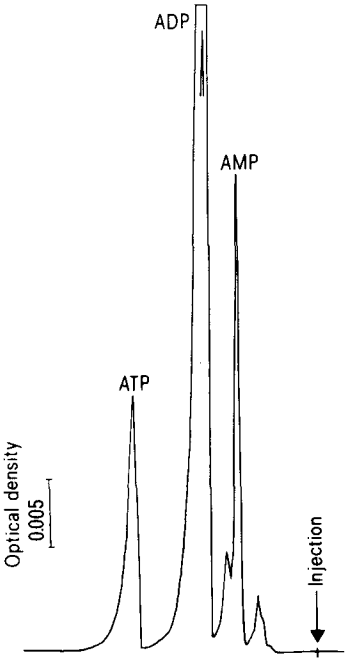
The exact mechanism of ATP synthesis during photophosphorylation still has not been fully elucidated. One hypothesis suggested that AMP might be phosphorylated to form a complex with the coupling factor and ADP[2,3], the latter being a kinetic intermediate in the formation of ATP. Recently, however, this hypothesis was rejected[4]. Several investigators proposed that bound nucleotides may play an intermediary role in the sequence of synthesis of free ATP[5-10]. On the other hand there is good evidence accumulating for a regulatory function of the tightly bound species[11-15]. Loose binding of ADP and ATP probably represents catalytic forms since they are only seen with an active coupling factor. According to the literature the number of tight binding sites for ADP is 1 or 2, and there is probably an additional binding site for ATP[2,17-19]. Kinetic experiments of the initial events of phosphorylation usually do not distinguish between bound and free nucleotides. However the lag between $^{32}P$ incorporation (5-20 msec)[20,21] and the formation of free ATP measured by luciferase (150 msec)[22] strongly suggests the presence of bound intermediates.

Most of the experiments investigating bound nucleotides have been done with isolated coupling factor which probably does not represent the actual situation on an intact membrane. It is the aim of the present investigation to analyze the firmly bound adenine nucleotides after various treatments of the chloroplast membranes.

## *Materials and methods*

Spinach chloroplasts were prepared according to Strotmann et al.[23]. Coupling factor 1, isolated from these membranes, was extracted using mainly the methods of Strotmann et al.[23] and Younis et al.[24]. By these procedures a pure enzyme fraction was obtained in much shorter time than with the methods of Lien and Racker[25] or Binder et al.[26], which were used for comparative purposes. The final purification in each case was obtained by sucrose gradient centrifugation (10-25% sucrose, 150 min at 55,000 rpm in a Ti-60 fixed angle rotor). A control with gel electrophoresis showed less than 5% protein impurities.

Phosphorylation was measured in a conventional PMS-system with batch volumes of 200 ml at a light intensity (white light, Comptalux 300 W) of $2 \cdot 10^5$ erg / $cm^2 \cdot$ sec and a chlorophyll concentration of 0.5 mg/ml (for composition of medium see tables). Adenine nucleotide exchange was done according to Strotmann[27]. Free nucleotides were then removed from the centrifuged membranes or isolated protein by gelfil-




Separation of adenine nucleotides by HPLC. 250 µl of extract from coupling factor were eluted with $(NH_4)H_2PO_4$ (0.4 M, pH 3.5) at a flow rate of 1 ml/min at room temperature. The pressure was 70 bars and the nucleotides measured at 257 nm.

tration (Sephadex G-50). The bound nucleotides were extracted from the membrane or from the isolated coupling factor by heat treatment, PCA combined with SDS according to Pflugshaupt[7] or with cold PCA (final concentration 4%) followed by centrifugation. These different procedures gave identical results. The analysis of the nucleotides was achieved by HPLC on a Nucleosil 10 SB column (Macherey-Nagel and Co.) of 4.5 × 250 mm size. Excess PCA was removed before chromatography by extraction with a mixture of Alamine 336 (General Mills) and freon[28]. The sample was eluted with 0.4 M $NH_4H_2PO_4$, pH 3.5. The absorption of the eluate was monitored at 257 nm and quantification was obtained by an electronic integrator. Control experiments showed that the recovery of the adenine nucleotides during the whole procedure was better than 95%. A typical separation is given in figure 1.

*Results and Discussion*

Table 1 gives the composition of the nucleotides bound to the coupling factor isolated from chloroplasts in the deenergized and energized state respectively. When coupling factor was isolated under strict dark conditions and in the absence of external nucleotides, approximately 2 moles of adenine nucleotides were found to be bound to 1 mole of coupling factor. The ADP accounted for about 60%, the ATP for 35% of the total nucleotides. In all experiments a small but substantial amount of bound AMP was also found. Even after the time used for isolation and purification of the enzyme no equilibration among the 3 nucleotides was observed, ruling out the action of myokinase on the bound nucleotides.

When chloroplasts were illuminated for 60 sec in the absence of nucleotides prior to the purification of the coupling factor, however, an increase of ATP and a concomitant decrease of ADP and AMP was seen, without a change in the total amount of bound adenine nucleotides. In the light, $^{32}P$ was incorporated into ATP and ADP in a ratio of 2.5:1, suggesting either a substantial exchange between ADP and P, or conversion of AMP to ADP in the light even without exchangeable nucleotides in the medium.

Incubation of membranes with free nucleotides in the medium enables the release and exchange of coupling factor bound nucleotides. It is shown in table 2 that the distribution under these conditions was quite similar to the one in the dark in the absence of an exchange. This pattern was largely independent of the presence of inhibitors like adenosine-5-pentaphosphate ($Ap_5A$) or phlorizin.

The results with the inhibitor of the adenylate kinase, $Ap_5A$, show that the distribution of the nucleotides is not influenced by the kinase which may still be present in washed chloroplasts (table 2). Furthermore, the energy transfer inhibitor phlorizin, which has no effect on the synthesis of bound nucleotides[7] changed neither the total amount nor the ratio of the nucleotides.

When incubated with low concentrations of $^{14}C$-labeled ADP, chloroplast membranes exchanged the bound nucleotides for labeled nucleotides of the medium in a light-dependent reaction (table 3). Yet this treatment changed neither the overall amount of bound nucleotides nor the ratio between ATP, ADP and AMP (table 3). The $^{14}C$ label was found mainly in the ADP, and to a small extent in ATP and AMP, probably as a result of an adenylate kinase-like

Table 1. Distribution of bound nucleotides after incubation of membranes in the dark and in the light in the absence of added adenine nucleotides (moles adenine nucleotides bound per mole coupling factor)

| | Dark | Light | Light $^{32}P$ distribution (%) |
|---|---|---|---|
| AMP | 0.10 | 0.05 | -- |
| ADP | 1.05 | 0.80 | 29 |
| ATP | 0.64 | 0.90 | 71 |
| Total | 1.79 | 1.75 | 100 |

Reaction conditions: Chloroplast fragments (1 mg Chl/ml), Tricine, pH 8.0 0.025 M, NaCl 10 mM, $MgCl_2$ 5 mM, BSA 1 mg/ml, Na-ascorbate 1 mM, PMS 0.05 mM, $Na_2PO_4$ (with $^{32}P$ in the light experiment) 1 mM, total volume 200 ml. Dark control: spinach leaves were kept in darkness for 15 h followed by the extraction of the coupling factor in darkness until separation from the membranes. Light incubation 60 sec.

Table 2. Distribution of bound nucleotides after incubation of chloroplast membranes in the light in the presence of added adenine nucleotides and inhibitors (moles bound adenine nucleotides per mole coupling factor)

| | Light control | + $Ap_5A$ | + Phlorizin |
|---|---|---|---|
| AMP | 0.06 | 0.05 | 0.05 |
| ADP | 1.21 | 1.30 | 1.20 |
| ATP | 0.70 | 0.60 | 0.79 |
| Total | 1.97 | 1.96 | 2.04 |

Reaction conditions: as in table 1, ADP 2 mM, $AP_5A$ 10 μM, phlorizin 5 mM.

Table 3. Distribution of bound nucleotides (moles adenine nucleotides bound per mole coupling factor) and $^{14}C$ label from $^{14}C$-ADP (in %) after a light-induced exchange reaction in the presence and absence of inhibitors

| | Light control | | + $Ap_5A$ | + Phlorizin | |
|---|---|---|---|---|---|
| | Bound nucleotides | $^{14}C$ label | Bound nucleotides | Bound nucleotides | $^{14}C$ label |
| AMP | 0.07 | 13% | 0.06 | 0.06 | 10% |
| ADP | 1.10 | 82% | 1.21 | 1.11 | 85% |
| ATP | 0.71 | 5% | 0.70 | 0.70 | 5% |
| Total | 1.88 | | 1.97 | 1.87 | |

Reaction conditions: as in table 1, ADP 2 mM (containing $^{14}C$-ADP), $Ap_5A$ 10 μM, phlorizin 5 mM.

reaction. As was already demonstrated for the phosphorylating system, neither $Ap_5A$ nor phlorizin had a significant effect on the ratio between ATP, ADP and AMP or on the incorporation of $^{14}C$-label from ADP.

The term 'bound' for nucleotides is based on the binding property of the nucleotides to the coupling factor and the stability of this interaction during purification. Although according to the literature, the number of bound nucleotides range from 1 to 4 nmoles/mg chlorophyll (equal to about 0.7–3 moles adenine nucleotides per mole of coupling factor) the most frequently cited number is two[5]. In the present study - assuming a ratio of 1 coupling factor per 860 chlorophylls - a value close to 2 nucleotides per coupling factor was found under all conditions. Yet, the distribution of AMP, ADP and ATP did not give integer numbers, possibly because the isolated coupling factor consists of a population made up of enzymes in different states having different nucleotide compositions, as has been discussed earlier by Gräber et al.[29].

Bound nucleotides are released into the medium during phosphorylation. This has been demonstrated with the light-induced exchange reaction which is possibly part of the phosphorylation sequence. The $^{32}P$ incorporation in the absence of exogenous nucleotides as well as the appearance of bound $^{14}C$-ADP from added free $^{14}C$-ADP show that the P moiety as well as the adenine ring system were exchanged in a light-dependent reaction possibly as a consequence of a conformational change of the protein. Small amounts of bound AMP, usually not described as a bound species, were formed on the coupling factor. In addition during a 60-sec light phosphorylation added $^{32}P$-phosphate was incorporated in the beta position of the ADP (table 1). This would point to a phosphorylation of AMP, a theory recently abandoned by Moudrianakis et al.[8]. The incorporation of $^{32}P$ into coupling factor bound ADP during phosphorylation was rather slow, only 0.7% of the bound ADP is labeled even in the absence of free nucleotides. Hence, this reaction can hardly be interpreted as supporting the notion that AMP is the primary phosphate acceptor. However, these experiments demonstrate clearly the presence of a transphosphorylase activity on the coupling factor which is not due to contamination of chloroplast myokinase.

These results also show that the chloroplast coupling factor has 2 sites for firmly bound nucleotides which are occupied mainly by ADP, but to a small extent also by ATP and AMP. This relaxed state is found both after a long dark incubation and in the light in the presence of external nucleotides.

1 Acknowledgment: We thank the Swiss National Foundation for Scientific Research (grant 3.582.79) for generous support.

2 Roy, H., and Moundrianakis, E.N., Synthesis and discharge of the coupling factor-ADP complex in spinach chloroplast lamellae. Proc. natl Acad. Sci. USA *68* (1979) 2720.
3 Tiefert, M.A., and Moudrianakis, E.N., Role of AMP in photophosphorylation by spinach chloroplasts. J. biol. Chem. *254* (1979) 9500.
4 Moudrianakis, E.N., Horner, R.D., and Tiefert, M.A., On the mechanism of ATP synthesis during photophosphorylation, in: Energy Coupling in Photosynthesis, p. 149. Eds B. Selman and S. Selman-Reimer. Elsevier/North-Holland, Amsterdam 1981.
5 Lutz, H.U., Dahl, J., and Bachofen, R., Synthesis of free ATP from membrane-bound ATP in chromatophores of *Rhodospirillum rubrum*. Biochim. biophys. Acta *347* (1974) 359.
6 Rosing, J., Harris, D.A., Slater, E.C., and Kemp, A., Possible role of tightly bound adenine-nucleotides in oxidative and photosynthetic phosphorylation. J. supramolec. Struct. *3* (1975) 284.
7 Pflugshaupt, Ch., and Bachofen, R., Bound ATP in chloroplast membranes: formation and effect of different inhibitors on the labelling. J. Bioenerg. *7* (1975) 49.
8 Moundrianakis, E.N., and Tiefert, M.A., Synthesis of bound ATP from bound ADP by the purified coupling factor 1 of chloroplasts. J. biol. Chem. *251* (1976) 7796.
9 Smith, D.J., Stockes, B.D., and Boyer, P.D., Probes of initial phosphorylation events in ATP synthesis by chloroplasts. J. biol. Chem. *251* (1976) 4165.
10 Harris, D.A., John, P., and Radda, G.K., Tightly bound nucleotides of the energy-transducing ATPase and their role in oxidative phosphorylation. Biochim. biophys. Acta *459* (1977) 546.
11 Shavit, N., Lien, S., and SanPietro, A., On the role of membrane-bound ADP and ATP in photophosphorylation in chloroplast membranes. FEBS lett. *73* (1977) 55.
12 Shoshan, V., Shavit, N., and Chipman, D.M., Kinetics of nucleotide binding to chlorplast coupling factor ($CF_1$). Biochim. biophys. Acta *504* (1978) 108.
13 Shavit, N., Energy transduction in chloroplasts: Structure and function of the ATPase complex. A. Rev. Biochem. *49* (1980) 111.
14 Shavit, N., Bar-Zvi, D., Podhajcer, O.L., and Aflalo, C., Role of tight nucleotide binding sites in the regulation of the chloroplast ATP synthetase functions, in: Proc. 5th Int. Photosynth. Congr. vol. 2, p. 789. Ed. G. Akoyunoglou. Balaban Int. Sci. Serv., Philadelphia 1981.
15 Bickel-Sandkötter, S., and Strotmann, H., Nucleotide binding and regulation of chloroplast ATP synthese. FEBS Lett. *125* (1981) 188.
16 Dunham, K.R., The modulation of the ATPase activity of membrane-bound $CF_1$ by ADP and phosphate, in: Energy Coupling in Photosynthesis, p. 209. Eds B. Seman and S. Selman-Reimer. Elsevier/North-Holland, Amsterdam 1981.
17 Cantley, L.C., and Hammes, G.G., Characterization of nucleotide binding sites on chloroplast coupling factor 1. Biochemistry *14* (1975) 2968.
18 Harris, D.A., and Slater, E.C., Thightly bound nucleotides of the energy-transducing ATPase of chloroplasts and their role in photophosphorylation. Biochim. biophys. Acta *387* (1975) 335.
19 Harris, D.A., The interactions of coupling ATPases with nucleotides. Biochim. biophys. Acta *463* (1978) 245.
20 Meier, A., and Bachofen, R., The temperature dependance of initial events in photophosphorylation of spinach chloroplasts. FEBS Lett. *127* (1981) 193.
21 Meier, A., Hofmann, M., and Bachofen, R., Initial events in photophosphorylation with chloroplasts and chromatophores: effects of temperature and of valinomycin, in: Energy coupling in Photosynthesis, p. 79. Eds B. Selman and S. Selman-Reimer. Elsevier/North-Holland, Amsterdam 1981.

22 Baltscheffsky, M., and Boork, J., Initiation of photophosphorylation in *Rhodospirillum rubrum* chromatophores, in: Proc. 5th Int. Photosynth. Congr., vol.2, p.929. Ed. G. Akoyunoglou. Balaban Int. Sci. Serv., Philadelphia 1981.
23 Strotmann, H., Hesse, H., and Edelmann, K., Quantitative determination of coupling factor $CF_1$ of chloroplasts. Biochim. biophys. Acta *314* (1973) 202.
24 Younis, H.M., Winget, G.D., and Racker, E., Requirement of the $\delta$ subunit of chloroplast coupling factor I for photophosphorylation. J. biol. Chem. *252* (1977) 1814.
25 Lien, S., and Racker, E., Preparation and assay of chloroplast coupling factor $CF_1$, in: Methods in Enzymology, vol.23. Eds S.P. Colowick and N.O. Kaplan. Academic Press, New York 1971.
26 Binder, A., Jagendorf, A.T., and Ngo, E., Isolation and composition of subunits of spinach chloroplast coupling factor protein. J. biol. Chem. *253* (1978) 3094.
27 Strotmann, H., and Bickel-Sandkötter, S., Energy-dependent exchange of adenine nucleotides on chloroplast coupling factor ($CF_1$). Biochim. biophys. Acta *314* (1973) 202.
28 Khym, J.X., An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. Clin. Chem. *21* (1975) 1245.
29 Gräber, P., Schlodder, E., and Witt, H.T., Conformational change of the chloroplast ATPase induced by a transmembrane electric field and its correlation to phosphorylation. Biochim. biophys. Acta *461* (1977) 426.

# Short Communications

## A new, unexpected marine source of a molting hormone. Isolation of ecdysterone in large amounts from the zoanthid *Gerardia savaglia*[1]

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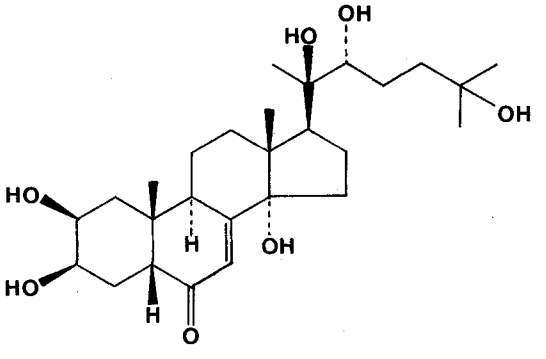
*Summary.* Ecdysterone was found in large amounts in the Mediterranean zoanthid *Gerardia savaglia* both immediately after its collection and after it had been kept for 15 months in an aquarium. This is the first time that an ecdysteroid has been found in a marine animal which does not belong to the phylum Arthropoda.

Several polyhydroylated steroidal $\Delta^7$-6 ketones have been initially isolated in extremely small amounts from both insects[5a] and crustaceans[5a,b] during ecdysis, and have hence been termed ecdysteroids. These molting hormones have also been found to occur in trace amounts in other terrestrial animals, such as nematodes[5c] and, usually in higher amounts, are also widely distributed in terrestrial plants[5a].

We report here that, during our search for natural antifungals[1], we have isolated ecdysterone (**1**) instead, in large quantities, from the zoanthid *Gerardia savaglia* Bert. (Cnidaria, Anthozoa, Hexacorallia, Zoanthidea).

The zoanthid, about 20 g dry wt, was collected in November 1980 in South Tyrrhenian, at a depth of 45 m, and immediately was steeped in ethanol. A small portion of the colony was maintained alive in an aquarium[6]. The zoanthid kept in ethanol was homogenized and twice extracted with fresh ethanol at room temperature. The combined extracts were evaporated at reduced pressure and the residue was partitioned first between water (100 ml) and ethyl ether (3 × 100 ml), and then between water and n-butanol (2 × 100 ml). The residue (2.2 g) after evaporation, at reduced pressure, of the butanol extract was subjected, one portion at a time, to reverse phase HPLC on a Jobin-Yvon Miniprep (25–40 µm RP-18, 50 g; methanol-water 1:1, 8 ml $min^{-1}$ for 35 min, under 10 at, monitoring at $\lambda = 254$ nm). The central fractions, on evaporation at reduced pressure, gave 0.061 g of colorless crystals which were further purified, practically without weight loss, by reverse phase




HPLC on a Merck 10 × 250 mm LiChrosorb RP-18,7-µm column, methanol-water 1:1. Recrystallization from methanol-ethyl acetate gave colorless crystals, m.p. 234–238 °C.

Spectral studies immediately revealed that the compound is an ecdysteroid and, in fact, both the above recrystallized and non-recrystallized samples were undistinguishable by either TLC or HPLC from commercially available ecdysterone (**1**) (Sigma). Also, our samples of **1** and commercially available **1** gave superimposable mass[7a], $^1$H-NMR[7c], $^{13}$C-NMR- and UV-spectra, as well as the same optical rotation. We report here both UV and optical-rotation data in greater details than was so far available[6b-d] as well as