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Photoaffinity cross-linking of F_1ATPase from spinach chloroplasts by 3′-arylazido-β-alanyl-8-azido ATP

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Abstract

UV irradiation of the ATPase (CF_1) from spinach chloroplasts in the presence of 3′-arylazido-β-alanyl-8-azido ATP (8,3′-DiN_3ATP) results in a nucleotide-dependent inactivation of the enzyme and in a nucleotide-dependent formation of α–β cross-links. The results demonstrate an interfacial localization of the nucleotide binding sites on CF_1.

Key words: F_1ATPase; Chloroplast; Photoaffinity cross-linking; Interfacial localization; Nucleotide binding site

1. Introduction

The catalytic part of the ATP synthase complex from chloroplasts (CF_1) is formed by three α-, three β-, one γ-, one δ- and one ε-subunit [1–4]. The number of nucleotide binding sites have been in debate for a long time. Based on results for photoaffinity labeling of CF_1 by 2-azido-adenine nucleotides three catalytic and three noncatalytic nucleotide binding sites are now believed to be present per CF_1 [5] in analogy to the F_1ATPases from mitochondria [6] and bacteria [7], but smaller numbers have been discussed too [8,9]. The position of these nucleotide binding sites could not be cleared finally. Photoaffinity labeling yielded the first indications for an interfacial localization of the nucleotide binding sites. Depending on the label applied, diverging results were obtained concerning the subunits labeled. The exclusive labeling of the β-subunits as well as an equal labeling of α and β have been reported [5,10–14]. Therefore the location of nucleotide binding sites on one subunit (for example β) in close proximity to a second subunit (for example α) looks plausible. Even the participation of both subunits in the formation of one nucleotide binding site seems possible [15,16]. An interfacial position of the nucleotide binding sites presents an attractive model to explain the strong cooperativity between the different catalytic sites postulated for the catalysis performed by ATP synthase complexes (for example the alternating site mechanism [17,18]). Such a model has been proposed, discussed and verified indirectly for different ATP synthases by several authors [16,19–32]. The first direct experimental proof for an interfacial location of the nucleotide binding sites of ATP synthase complexes (F_1,F_0F_1) from bacteria and mitochondria has been obtained by photoaffinity cross-linking of these enzymes by bifunctional photoactivatable ATP analogs like 8,3′-DiN_3ATP [15,33–35], 2,3′-DiN_3ATP [36,37] or FSB-8-N_3A [38,39]. The results are principally analoguous, despite some small differences in the cross-links yields or in the cross-links patterns on SDS electrophoresis gels, obviously due to the different conformations of 2- or 8-substituted azidoadenine nucleotides or to the different affinities of the labels to the catalytic and noncatalytic nucleotide binding sites. In all these experiments α–β cross-links were formed as the main product proving the interfacial position of nucleotide binding sites between α and β. Up to now this direct indication has not been achieved for the chloroplast coupling factor. Here we report on photoaffinity cross-linking of the F_1ATPase from spinach chloroplasts.

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Abbreviations: CF_1, coupling factor 1 (ATPase) from chloroplasts; F_1, F_1ATPase from mitochondria or bacteria; F_0F_1, F_0F_1ATPase, ATP synthase; 2,3′-DIN_3ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}2-azidoadenosine 5-triphosphate; 8,3′-DiN_3ATP, 3′-aryl-azido-β-alanyl-8-azido ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}8-azidoadenosine 5-triphosphate; FSB-8-N_3A, 5′-*p*-fluorosulfonylbenzoyl-8-azidoadenosine; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-adenosine 5′-triphosphate.

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2. Materials and methods

2.1. Preparation of F_1ATPase (CF_1) from spinach chloroplasts

CF_1 was prepared by EDTA extraction from market spinach as described by Lien and Racker [40] with the exception that DEAE Sepharose CL-6B (Pharmacia) was used instead of DEAE Sephadex A-50. ATPase activity was determined by continuous measurement of the liberated phosphate at 37°C in 5 ml test solution containing 2 μg CF_1, 25 mM Tricine (pH 8.0), 33% methanol, 2 mM Ca^{2+} and 1 mM ATP [41]. Protein concentrations were measured according to Lowry et al. [42].

2.2. Photoaffinity cross-linking

8,3'-DiN_3ATP was synthesized by esterification of *N*-4-azido-2-nitrophenyl-β-alanine with 8-azido ATP [43] as described earlier [33,44] according to Jeng and Guillory [45]. Photoaffinity cross-linking was performed by irradiation (λ = 310 nm) of CF_1 (usually 50–100 μg) in 500 μl Tricine/HCl buffer (25 mM, pH 8.0) with a Xenon-lamp (Osram *XBO, 450 W*) and a high-intensity monochromator (Bausch and Lomb, Cat. No. 33-86-79) in the presence of Mg · 8,3'-DiN_3ATP (20 μM) at 37°C. The separation of the cross-linked CF_1 subunits was performed by SDS-PAGE on 10–15% T (w/v) gels according to Lämmli [46].

2.3. Immunization of rabbits

Immunization of rabbits was done according to Berzborn [47].

2.4. Western immuno blot

Western immuno blots were carried out essentially as described by Towbin et al. [48], but using horse radish peroxidase conjugated 2nd antibody.

3. Results and discussion

Irradiation of CF_1 in the presence of 8,3'-DiN_3ATP resulted in an inactivation of the ATPase (Fig. 1). This inactivation was observed neither by irradiation of CF_1 in the absence of the label nor by incubation of CF_1 in the presence of 8,3'-DiN_3ATP in the dark, except for a small inactivation in the dark obviously due to noncovalent interactions; this may be caused by the additional hydrophobic phenyl ring of the label as compared with ATP. Such increased interactions have often been described for aromatic 2'- or 3'-substituted adenine nucleotides like 2',3'-*O*-(2,4,6-trinitrophenyl)-adenosine 5'-triphosphate (TNP-ATP) [49,50]. Those analogs usually show a much higher affinity to F_1ATPases than ATP, resulting in a strong competitive inhibition of the enzyme [51].

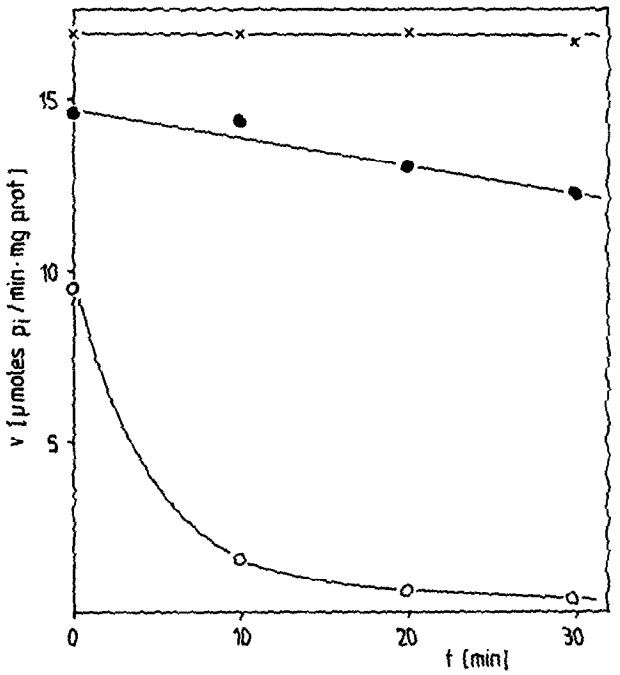

Fig. 1. Light-induced inhibition of CF_1 by 8,3'-DiN_3ATP. Irradiation in the presence of 20 μM Mg · 8,3'-DiN_3ATP (○), dark control in the presence of 20 μM Mg · 8,3'-DiN_3ATP (●), light control in the absence of 8,3'-DiN_3ATP (×). ATPase activity was determined at 37°C in 5 ml test solution containing 2 μg CF_1, 25 mM Tricine (pH 8.0), 33% methanol, 2 mM Ca^{2+} and 1 mM ATP.

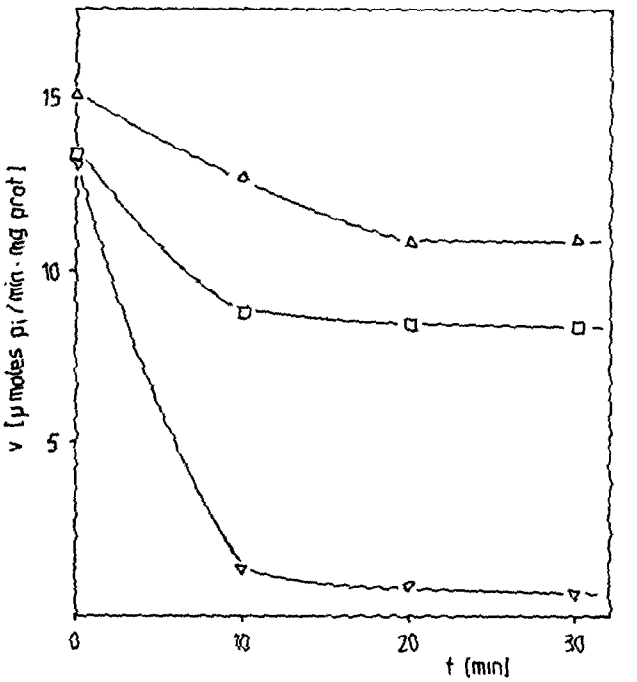

Fig. 2. Effect of added Mg · nucleotides on light-induced inhibition of CF_1 by 8,3'-DiN_3ATP. Irradiation of CF_1 in the presence of 20 μM Mg·8,3'-DiN_3ATP and 1 mM Mg·AMP (▽), 1 mM Mg·ADP (△) or 1 mM Mg·ATP (□). ATPase activity was determined as described in Fig. 1.

The light-induced inhibition of CF_1 by 8,3'-DiN_3ATP could be prevented to a high degree by the addition of ATP or ADP prior to the labeling procedure (Fig. 2). Both nucleotides compete with the label for the nucleotide binding sites. The addition of AMP which does not interact specifically with these sites did not influence the inactivation by photoaffinity labeling. This indicates that the inactivation was caused by the covalent labeling of the nucleotide binding sites by 8,3'-DiN_3ATP.

In addition photoaffinity labeling of CF_1 by the bifunctional diazidoadenine nucleotide resulted in the formation of higher molecular mass cross-links (Fig. 3). This is analoguous to the results obtained with different solubilized ATP synthase complexes from bacteria or mitochondria [15,33–39].

Corresponding to the results on the light-induced inactivation the formation of these cross-links could be decreased by the addition of ATP or ADP (Fig. 3B). The presence of AMP during photoaffinity labeling did not influence the cross-links yields. These experiments prove the involvement of nucleotide binding sites in the formation of the cross-links.

The subunit composition of the CF_1 cross-link was determined by immunological analysis (Fig. 4). The formed cross-link interacted specifically with antibodies against the α and the β subunits of CF_1, not with the antiserum against γ. Antibodies against the smaller subunits δ and ε also did not interact with the cross-link (data not shown). This demonstrates the subunit composition α–β for this cross-link for the first time by a specific immunochemical method, confirming the results on photoaffinity cross-linking of the bacterial and mitochondrial F_1ATPases which had been obtained by hydrolytic cleavage of the cross-links and a subsequent electrophoretical separation of the cleavage products [15,35–38,44].

In addition to the two-subunit cross-links even higher molecular mass cross-links could be observed upon the application of higher amounts of labeled CF_1 onto the SDS gels (not shown). These cross-links are probably formed by three of the α and/or β subunits. This result would be in agreement with the localization of all the catalytic and noncatalytic nucleotide binding sites at α–β interfaces. Such a model has been proposed by Schäfer et al. [35,36] for the F_1ATPase from the thermophilic bacterium PS3 and recently by Gromet-Elhanan [16] for the enzyme from *Rhodospirillum rubrum*.

In summary, the interfacial localization of the nucleotide binding sites seems to be a common feature for the structure of the catalytic F_1 complex of the ATP synthases from bacteria, mitochondria and chloroplasts.

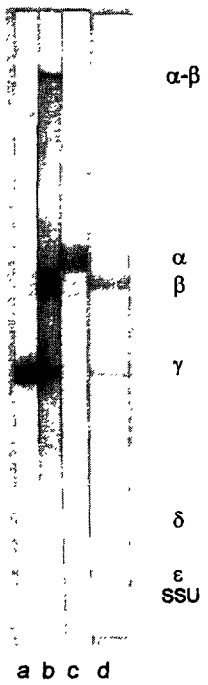

Fig. 4. Specific immunochemical analysis of cross-linked (labeled by 20 μM Mg · 8,3′-DiN_3ATP) CF_1 by Western blot: (a–c) interaction with antibodies against CF_1 subunits: anti-CF_1-γ (a), anti-CF_1-β (b), anti-CF_1-α (c); (d) stained for protein (Amido black). Designation of CF_1 subunits and cross-link products as in Fig. 3.

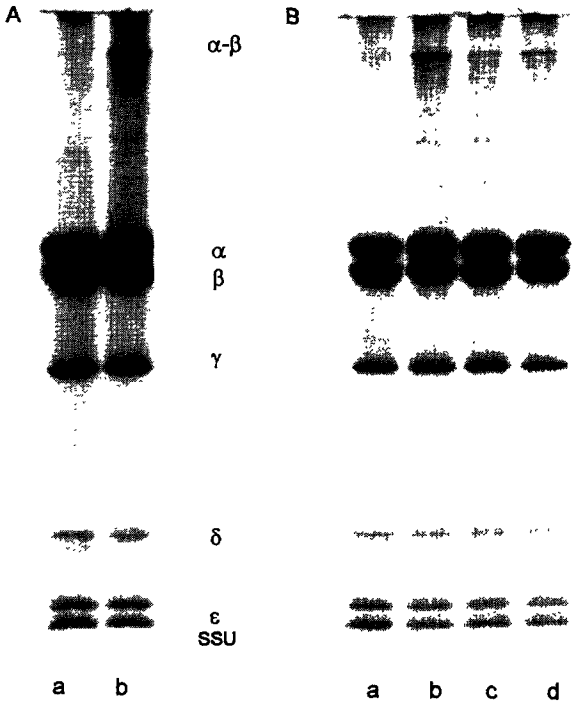

Fig. 3. Photoaffinity cross-linking of CF_1 by 8,3′-DiN_3ATP. SDS electrophoresis gels of labeled (cross-linked) CF_1: (a) native CF_1 (25 μg), (b) CF_1 (25 μg) labeled by 20 μM Mg·8,3′-DiN_3ATP. (B) Effect of added Mg·nucleotides on photoaffinity cross-linking of CF_1 by 8,3′-DiN_3ATP. SDS electrophoresis gels: (a) native CF_1 (15 μg), (b–d) CF_1 (15 μg) labeled by 20 μM Mg·8,3′-DiN_3ATP in the presence of 1 mM Mg·AMP (b), 1 mM Mg·ADP (c) or 1 mM Mg·ATP (d). α, β, γ, δ, ε, subunits of CF_1; α–β, cross-link product; SSU, small subunit of ribulose-bisphosphate carboxylase.

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