2,8-Diazido-ATP — a short-length bifunctional photoaffinity label for photoaffinity cross-linking of a stable F_1 in ATP synthase (from thermophilic bacteria PS3)

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Abstract To demonstrate the direct interfacial position of nucleotide binding sites between subunits of proteins we have synthesized the bifunctional photoaffinity label 2,8-diazidoadenosine 5′-triphosphate (2,8-DiN$_3$ATP). UV irradiation of the F_1-ATPase (TF_1) from the thermophilic bacterium PS3 in the presence of 2,8-DiN$_3$ATP results in a nucleotide-dependent inactivation of the enzyme and in a nucleotide-dependent formation of α-β cross-links. The results confirm an interfacial localization of all the nucleotide binding sites on TF_1.

Key words: F_1-ATPase; Short-length bifunctional photoaffinity label; Photoaffinity cross-linking; Direct interfacial localization; Nucleotide binding site

1. Introduction

F_1-ATPases [1–4], the catalytic parts of ATP synthase complexes, are formed by three α, three β, one γ, one δ and one ε subunit containing three catalytic and three noncatalytic nucleotide binding sites [5]. An interfacial position of these nucleotide binding sites between α and β presents an attractive model to explain the strong cooperativity between all three catalytic sites of ATP synthases postulated for the binding change mechanism [6]. The interfacial position of the binding sites has been proposed for different ATP synthases by several authors [7–12]. This could be demonstrated by X-ray analysis for the enzyme from beef heart mitochondria [13]. The first direct experimental proof, however, for an interfacial location of the nucleotide binding sites of ATP synthase complexes (F_1, F_0F_1) from bacteria, mitochondria and chloroplasts has already been obtained by photoaffinity cross-linking of these enzymes by bifunctional photoactivatable ATP analogs like 8,3′-DiN$_3$ATP [14–18], 2,3′-DiN$_3$ATP [19,20] or FSB-8-N$_3$A [21,22]. The great distance of both the reactive groups (ca. 2 nm) in all the labels used until now, however, is disadvantageous for an unambiguous evidence of such an interfacial localization. Cross-linking can even occur when the distance between the nucleotide binding site isolated on one subunit and the adjacent second subunit is up to 2 nm. To exclude this disadvantage we have introduced two photoactivatable azido groups directly into the positions 2 and 8 of the adenine ring of ATP. Upon UV irradiation of 2,8-diazidoadenosine 5′-triphosphate (2,8-DiN$_3$ATP) these two azido groups form highly reactive nitrenes that react rapidly with amino acid residues located directly in the nucleotide binding site or in its nearest vicinity. Here we describe the synthesis of 2,8-DiN$_3$ATP and its first application on photoaffinity cross-linking of F_1-ATPase from the thermophilic bacterium PS3 (TF_1). In contrast to other F_1-ATPases the $\alpha_3\beta_3$ and $\alpha_1\beta_1$ complexes of TF_1 are active and stable [23]. The $\alpha_1\beta_1$ follows simple Michaelis-Menten kinetics with a K_m value of 70 μM [24]. The $\alpha_3\beta_3$ crystal free from nucleotide (3-fold symmetrical, space group P3$_1$2 [25]) has been analyzed in cooperation with Dr. Y. Shirakihara and Dr. J.E. Walkers laboratory (the results will be reported). Thus, the cross-link study of the native TF_1 will be useful in the future developments.

2. Materials and methods

2.1. 2,8-Diazidoadenosine-5′-triphosphate (2,8-DiN$_3$ATP)

2,8-DiN$_3$ATP was prepared in analogy to the synthesis of 8-azidoadenosine 5′-triphosphate (8-N$_3$ATP) [26] in a two step procedure starting with 2-azidoadenosine 5′-triphosphate (2-N$_3$ATP) via 2-azido-8-bromoadenosine 5′-triphosphate (2-N$_3$-8-BrATP) (Fig. 1). 2-N$_3$ATP was synthesized as described by van Dongen et al. [27] according to Schaeffer and Thomas [28], Sowa and Ouchi [29], and Hoard and Ott [30].

2.1.1. 2-N$_3$-8-BrATP. 2-N$_3$ATP triethylammonium salt (170 mg, 0.2 mmol) was dissolved in 2 ml potassium acetate buffer (1 M, pH 3.9). Then 15.5 μl (0.3 mmol) bromine were added. The solution was kept in the dark at room temperature for 5 h. During that time the absorption maximum shifted from 270 nm to 275 nm. Then the surplus bromine was reduced by adding small portions of sodium disulfite ($Na_2S_2O_5$) until the solution became colourless. 2-N$_3$-8-BrATP was precipitated by the addition of 50 ml of ice-cold ethanol. After standing at 0°C for 1 h the precipitated nucleotide was collected by centrifugation and redissolved in 5 ml of water. Further purification was performed by anion-exchange chromatography on a DEAE-Sephadex A-25 column (1.8 × 45 cm) with a linear gradient of 750 ml each of 0.2 M and 0.7 M triethylammonium bicarbonate (pH 7.5). Only the fractions absorbing maximally at 275 nm were collected and pooled to exclude a contamination by unreacted 2-N$_3$ATP. 2-N$_3$-8-BrATP was obtained as triethylammonium salt by freeze drying.

2.1.2. 2,8-DiN$_3$ATP. Intensively dried 2-N$_3$-8-Br-ATP triethylammonium salt (0.15 mmol) was dissolved in freshly distilled dimethylformamide (3 ml). Then 1.2 ml of a dry solution of HN_3 (1 M) in

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Abbreviations: F_0F_1, F_0F_1-ATPase, ATP synthase from mitochondria, bacteria or chloroplasts; F_1, F_1-ATPase; TF_1, F_1-ATPase from the thermophilic bacterium PS3; CF_1, coupling factor 1 (F_1-ATPase) from chloroplasts; 2-N$_3$ATP, 2-azidoadenosine 5′-triphosphate; 8-N$_3$ATP, 8-azidoadenosine 5′-triphosphate; 2-N$_3$-8-BrATP, 2-azido-8-bromoadenosine 5′-triphosphate; 2,8-DiN$_3$ATP, 2,8-diazidoadenosine 5′-triphosphate; 2,3′-DiN$_3$ATP, 3′-arylazido-β-alanyl-2-azido-ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}2-azidoadenosine 5′-triphosphate; 8,3′- DiN$_3$ATP, 3′-arylazido-β-alanyl-8-azido-ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}8-azidoadenosine 5′-triphosphate; FSB- 8-N$_3$A, 5′-*p*-fluorosulfonylbenzoyl-8-azidoadenosine.

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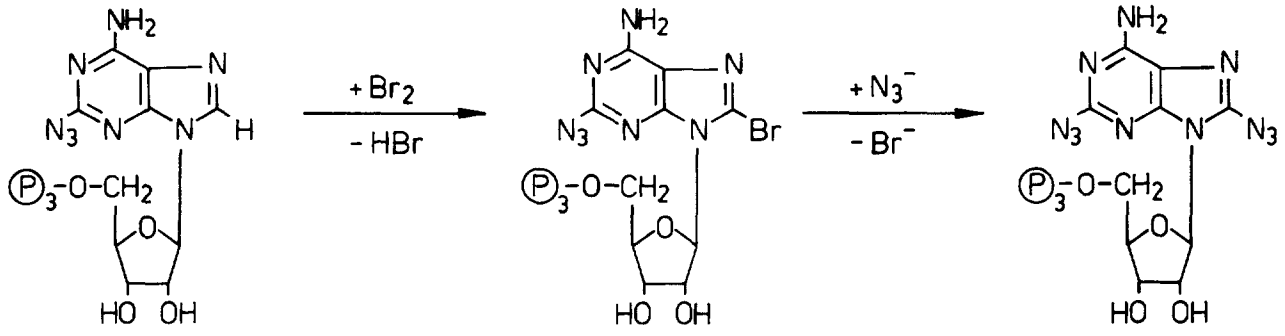

Fig. 1. Synthesis of 2,8-DiN_3ATP.

benzene and 0.17 ml triethylamine were added. The reaction mixture was allowed to stand at 75–80°C in the dark for 6 h. During that time the absorption maximum shifted from 277 nm to 297 nm. After completion of the reaction the solvent was evaporated under vacuum. The residue was redissolved in 3 ml of water. Further purification was acchieved by anion-exchange chromatography (DEAE-Sephadex A-25) as described above. In addition to 2,8-DiN_3ATP the mono- and the diphosphate of the bifunctional diazidoadenosine were eluted before. The fractions were collected and lyophilized. All the nucleotides resulted as trietylammonium salts. They can be stored at −20°C in the dark for month.

2.2. Preparation of F_1-ATPase (TF_1) from the thermophilic bacterium PS3

TF_1 was prepared from plasma membranes of PS3 as described earlier [31]. The absence of tightly bound nucleotides was tested by HPLC after acid denaturation, UV absorption or phosphate analysis. The protein concentration was measured according to Lowry et al. [32]. ATPase activity was determined by continuous measurement of the liberated phosphate at 60°C in 5 ml of test solution containing 0.5 μg TF_1, 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} and 1 mM ATP [33].

2.3. Photoaffinity cross-linking

Photoaffinity cross-linking was performed by irradiation (λ = 310 nm) of TF_1 (usually 100 μg) in 500–1000 μl Tris-HCl buffer (100 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·2,8-DiN_3ATP (0.5 mM) at 37°C. The separation of the cross-linked TF_1 subunits was performed by SDS-polyacrylamide gel electrophoresis on 10–15% T (w/v) gels according to Lämmli [34].

2.4. Western immuno blots

The antibodies against the subunits of TF_1 were prepared as described by Yoshida et al. [35]. Western immuno-blots were carried out essentially as described by Towbin et al. [36], but using horseradish peroxidase conjugated 2nd antibody.

3. Results and discussion

3.1. Synthesis and characterization of 2,8-DiN_3ATP

Fig. 1 shows the synthesis of the bifunctional photoactivatable 2,8-DiN_3ATP. The second azido-group was introduced into the monofunctional 2-N_3ATP by bromination and the subsequent exchange of the bromine for the azido function at position 8 of the adenine ring analogous to the synthesis of 8-N_3ATP [26]. 2,8-DiN_3ATP was characterized by IR-, ^1H NMR- and UV-spectroscopy. The IR spectrum shows an intensive band at 2140 cm^{-1} with a distinct shoulder at 2170 cm^{-1}. IR bands at these positions are characteristic for N_3 stretching vibrations. In addition no ^1H NMR signal could be detected for hydrogen atoms at the positions 2 and 8 of the purine ring. The UV absorption spectrum of 2,8-DiN_3ATP in aqueous solution shows a maximum at 307 nm and two shoulders at 318 nm and 333 nm. The UV spectrum strongly depends on the pH value and on the polarity of the solvent. This drastic change in UV absorbance is typical for 2-azidoadenine derivatives due to their azidoazomethine-tetrazole tautomerism [37]. The adjustment of the azidoazomethine–tetrazole equilibrium occurred relatively slowly. It could be accelerated significantly by increasing the temperature. The photolytic activity of 2,8-DiN_3ATP was demonstrated by photolysis of the compound which was observed spectroscopically (Fig. 2). Irradiation (λ = 310 nm) in aqueous solution resulted in the decomposition of the analog

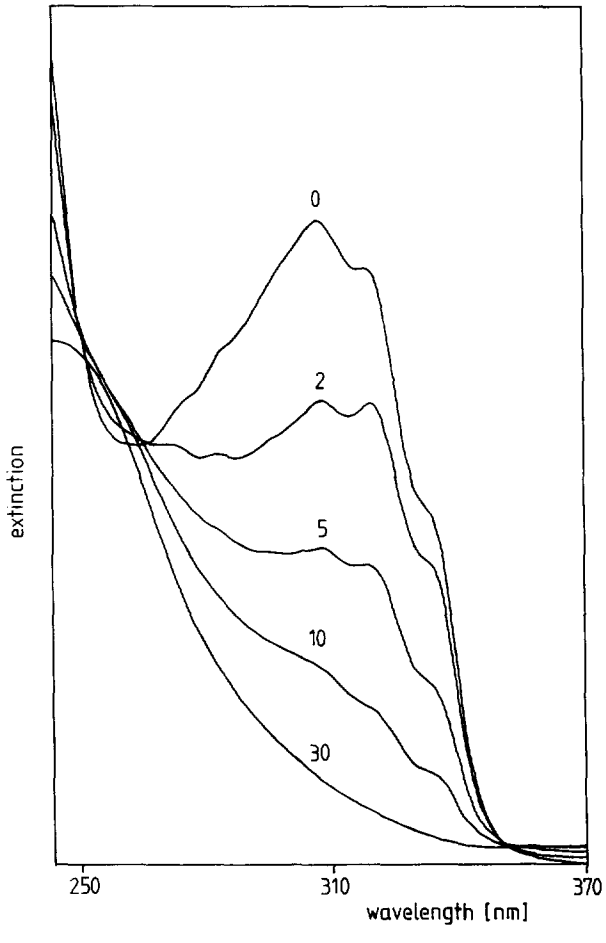

Fig. 2. Change of the optical absorption spectrum of 2,8-DiN_3ATP (aqueous solution) upon irradiation with UV light (λ = 310 nm) at 20°C. Irradiation time: 0, 2, 5, 10 and 30 min (0 = unirradiated control).

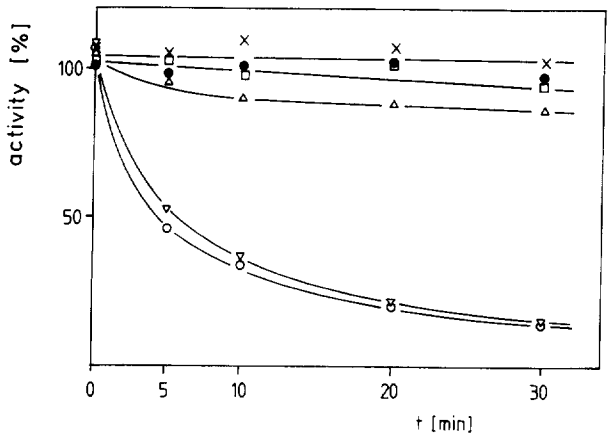

Fig. 3. Light-induced inhibition of TF_1 by 2,8-DiN_3ATP. Irradiation in the presence of 0.5 mM Mg·2,8-DiN_3ATP (○), dark control in the presence of 0.5 mM Mg·2,8-DiN_3ATP (●), light control in the absence of 2,8-DiN_3ATP (×). Effect of added Mg·nucleotides on light-induced inhibition of TF_1 by 2,8-DiN_3ATP. Irradiation of TF_1 in the presence of 0.5 mM Mg·2,8-DiN_3ATP and 1 mM Mg·AMP (▽), 1 mM Mg·ADP (△) or 1 mM Mg·ATP (□). ATPase activity was determined at 60° C in 5 ml test solution containing 0.5 μg TF_1, 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} and 1 mM ATP.

as demonstrated by the disappearance of the UV absorbance between 250 nm and 350 nm. All these results verify the structure of 2,8-DiN_3ATP.

3.2. Specific interaction of 2,8-DiN_3ATP with TF_1

The specific interaction of the label with the investigated protein is an essential precondition for a useful application of a ligand analog for affinity and photoaffinity labeling. This precondition for a suitable photoaffinity label for enzymes is fulfilled best if the label is a substrate or at least a competitive inhibitor, both in the dark. TF_1 hydrolyzed 2,8-DiN_3ATP in the presence of Mg^{2+} ions (hydrolysis rate: 3.0 μmol/min·mg protein). This rate is identical with the hydrolysis rate for Mg 8-N_3ATP indicating that 2,8-DiN_3ATP obviously prefers the *syn* conformation like 8-substituted ATP analogs and not the *anti* conformation like ATP or 2-substituted ATP analogs [38].

3.3. Light-induced inactivation of TF_1 by 2,8-DiN_3ATP

Irradiation of TF_1 in the presence of 2,8-DiN_3ATP and Mg^{2+} ions resulted in the nucleotide-specific decrease of ATPase activity (Fig. 3). This inactivation occurred neither by irradiation of TF_1 in the absence of the label nor by incubation of TF_1 in the presence of 2,8-DiN_3ATP in the dark. Addition of competing nucleotides like ATP or ADP prior to the labeling procedure prevented the light-induced inhibition of TF_1 by 2,8-DiN_3ATP almost completely. The addition of AMP, however, which does not interact specifically with the nucleotide binding sites of TF_1, did not influence the rate of light-induced inhibition by 2,8-DiN_3ATP at all. These results demonstrate the specific covalent labeling of nucleotide binding sites of TF_1 by the newly synthesized bifunctional photoaffinity label.

3.4. Photoaffinity cross-linking of TF_1 subunits by 2,8-DiN_3ATP

In addition to the light-induced inhibition, photoaffinity labeling of TF_1 by 2,8-DiN_3ATP resulted in the formation of α–β cross-links as demonstrated by immunological analysis (Figs. 4 and 5). The formed cross-links interacted specifically with antibodies against the α and the β subunits of TF_1 (*anti*-TF_1-α and *anti*-TF_1-β) exclusively. Antibodies against the smaller subunits did not interact with the cross-links (data not shown). In agreement with the results on light-induced inactivation of TF_1 by 2,8-DiN_3ATP the addition of ATP and ADP prevented the formation of the cross-links whereas the addition of AMP did not affect the cross-links' yield.

These cross-links are analoguous to the α–β cross-links obtained by photoaffinity labeling of different F_1-ATPases from bacteria, mitochondria or chloroplasts using other bifunctional azidoadenine nucleotides like 8,3′-DiN_3ATP, 2,3′-DiN_3ATP or FSB-8-N_3A [14–22]. Compared with these labels, however, the distance of both reactive groups in 2,8-DiN_3ATP is so short to ensure an exclusive cross-linking of α and β subunits only upon the direct positioning of the nucleotide binding site at the α–β interface.

The discrepancy between the rate of inactivation (about 80%) and the amount of formed cross-links (only few percents) is easy to explain. Due to the rigid structure of the purine there is no flexibility concerning the position of the nitrenes in the highly reactive intermediate formed upon irradiation of 2,8-DiN_3ATP. This contrasts to the flexible positions of both reactive groups in the 3′-arylazido-azidoadeninenucleotides like 2,3′-DiN_3ATP or 8,3′-DiN_3ATP. The rigid orientation of 2,8-DiN_3ATP resulted in the formation of cross-links only when the label fitted exactly to the binding site. Otherwise the main part of the label is linked only once to the protein. Inactivation,

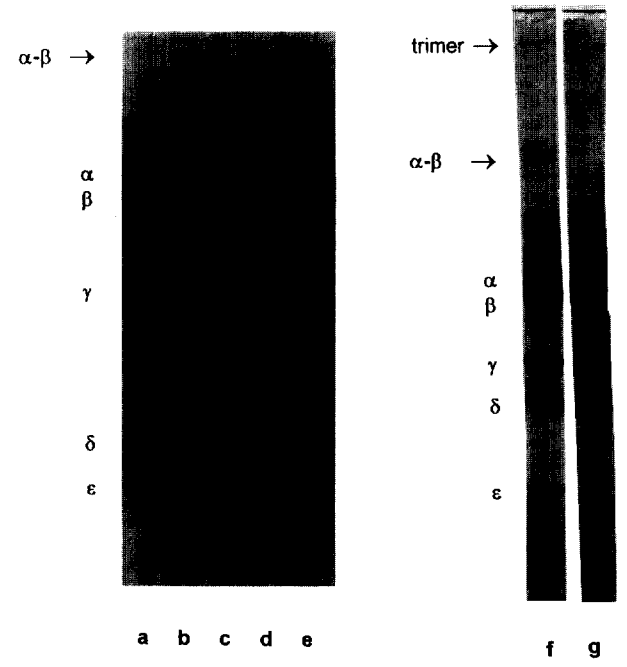

Fig. 4. Photoaffinity cross-linking of TF_1 and Ml-F_1 by 2,8-DiN_3ATP. SDS electrophoresis gels of labeled (cross-linked) TF_1: native TF_1 (25 μg) (a), TF_1 (25 μg) labeled by 0.5 mM Mg·2,8-DiN_3ATP (b), (c–e) effect of added Mg·nucleotides on photoaffinity cross-linking of TF_1 by 2,8-DiN_3ATP. TF_1 (25 μg) labeled by 0.5 mM Mg·2,8-DiN_3ATP in the presence of 1 mM Mg·ATP (c), 1 mM Mg·ADP (d) or 1 mM Mg·AMP (e). SDS electrophoresis gels (5–15%) of labeled (cross-linked) Ml-F_1: Ml-F_1 labeled by 0.5 mM Mg·2,8-DiN_3ATP (f), native Ml-F_1 (g).

however, was caused by a single labeling of the catalytic site as well as by a double one.

Summarizing, the nucleotide-specific formation of α–β cross-links upon irradiation in the presence of 2,8-DiN_3ATP nevertheless proves unambiguously a direct interfacial position of nucleotide binding sites in TF_1 despite the poor yield obtained.

In addition to the two-subunit cross-links small amounts of even higher molecular mass cross-links could be detected in some experiments because there are two kinds of nucleotide binding sites — three catalytic and three noncatalytic — between the α and β subunits [13]. These cross-links are analogous to the cross-links observed with 2,3′- and 8,3′-DiN_3ATP [17–19]. They are probably formed by three of the major subunits α and β (trimer). Fig. 4 (lanes f and g) shows the result obtained upon photoaffinity cross-linking of F_1-ATPase from *Micrococcus luteus* (Ml-F_1). This result confirms the localization of all the nucleotide binding sites at α–β interfaces as postulated by Schäfer et al. [17–19] (Fig. 6) for TF_1 and CF_1 and by Gromet- Elhanan [11] for the enzyme from *Rhodospirillum rubrum*. The interfacial localization of all the six catalytic and noncatalytic nucleotide binding sites was demonstrated impressively by X-ray analysis of the enzyme from beef heart mitochondria by Abrahams et al. [13] in which the catalytic sites

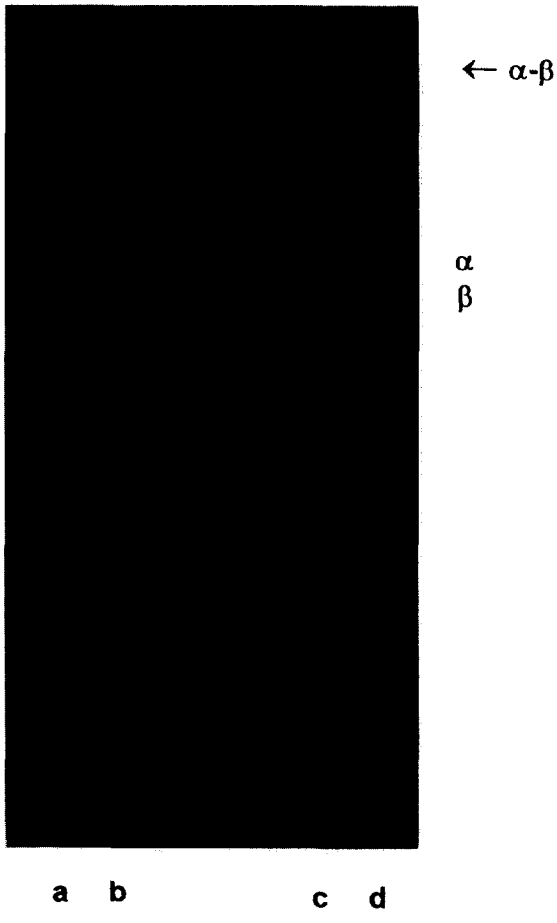

Fig. 5. Specific immunochemical analysis of cross-linked (labeled by 0.5 mM Mg·2,8-DiN_3ATP) TF_1 by Western blot. Interaction of TF_1 with antibodies against TF_1 subunits (a–d): native TF_1/anti-TF_1-α (a), native TF_1/anti-TF_1-β (b), cross-linked TF_1/anti-TF_1-α (c) and cross-linked TF_1/anti-TF_1-β (d).

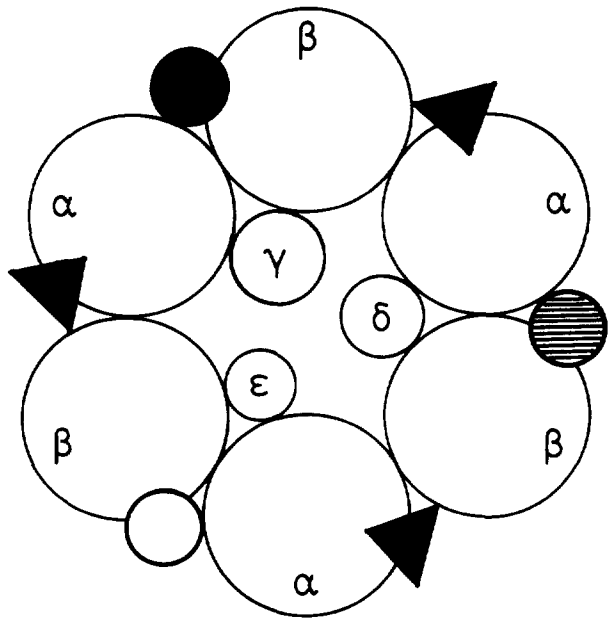

Fig. 6. Interfacial localization of nucleotide binding sites on F_1-ATPases (schematic): catalytic nucleotide binding sites (○, hatched circle, ●, representing the open, loose and tight binding sites, respectively, according to the binding change mechanism [6]), noncatalytic nucleotide binding sites (▲). The positions of γ, δ and ε are arbitrary.

were mainly positioned at the β and the noncatalytic sites mainly at the α subunits.

All results verify the suitability of 2,8-DiN_3ATP for an experimental proof of a direct interfacial position of nucleotide binding sites of proteins.

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