

Photoaffinity cross-linking of F_1 ATPase from the thermophilic bacterium PS3 by 3'-arylazido- β -alanyl-8-azido ATP

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To study the localization of the nucleotide binding sites of coupling factor 1 (TF_1) from the thermophilic bacterium PS3 we used the bifunctional (cross-linking) 3'-arylazido- β -alanyl-8-azido ATP (DiN_3ATP) for photoaffinity labeling. DiN_3ATP is hydrolyzed by TF_1 in the absence of ultraviolet light. Irradiation (UV light) of TF_1 in the presence of DiN_3ATP results in a nucleotide-specific reduction of ATPase activity and in a nucleotide-specific formation of different cross-linked proteins (dimers, trimers, oligomers) formed by the major subunits α and/or β . The results suggest that nucleotide binding sites (one, two, possibly all) are located at the interfaces between these subunits.

Bacterial F_1 ATPase	Thermophilic bacterium PS3	Photoaffinity cross-linking	Nucleotide-binding site
		α/β interface	

1. INTRODUCTION

Photoaffinity labeling of different F_1 ATPases by monovalent azido nucleotides resulted in diverse labeling with respect to the enzyme subunits [1–12]. It led to either a preferential labeling of the β subunits, or to an almost equal labeling of α and β , or to a preferential labeling of α . These discrepancies could be caused by different labeling conditions employed, different origins of the enzymes which contain different amounts of firmly bound nucleotides and Mg^{2+} , or different structures of the photoaffinity labels applied. However, it is also possible that the nucleotide binding sites are located at the interfaces between the major subunits α and/or β . In this case

monovalent azido ATP analogs can label either the α or β subunit. The labeling depends on the exact orientation of the photosensitive nucleotide at the binding site between the two subunits. To test this possibility we have synthesized the bifunctional DiN_3ATP [13].

If the nucleotide binding site is localized at or near the interface, the two nitrene groups formed upon irradiation of DiN_3ATP should partially react with amino acid residues of different subunits. The nitrene at position 8 of the adenine ring may react immediately at the adenine binding site, whereas the nitrene group produced from the 3'-arylazido group may react at locales more distant from the adenine binding site. Photoaffinity labeling by DiN_3ATP should result in the nucleotide specific formation of cross-links of neighboring subunits (photoaffinity cross-linking), containing the nucleotide binding site at their interface. Thus, photoaffinity cross-linking of F_1 ATPase from *Micrococcus luteus* by DiN_3ATP led to the formation of an α - β cross-link [14].

Abbreviations. TF_1 , coupling factor 1 (F_1 ATPase) from the thermophilic bacterium PS3, DiN_3ATP , 3'-arylazido- β -alanyl-8-azido ATP, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl}-8-azido-adenosine 5'-triphosphate; 8- N_3ATP , 8-azido ATP, 8-azido-adenosine 5'-triphosphate

Upon irradiation of oligomycin-sensitive ATPase from beef heart mitochondria in the presence of DiN_3ATP a higher molecular mass cross-link (>100 kDa) was also observed. This cross-link is supposedly analogous to the α - β cross-link from the bacterial enzyme [15].

Here we report on photoaffinity cross-linking of the coupling factor 1 from the thermophilic bacterium PS3 by irradiation in the presence of DiN_3ATP . The reason why TF_1 was used is (i) it does not contain firmly bound nucleotides and Mg^{2+} , (ii) it is very stable against nonspecific chemical treatment and (iii) it is reconstitutible from its subunits after denaturation with SDS [16].

2. MATERIALS AND METHODS

2.1. Preparation of F_1ATPase from the thermophilic bacterium PS3

TF_1 was prepared from plasma membranes of PS3 as described [12,16]. The absence of bound nucleotides and Mg^{2+} in TF_1 used was confirmed. The enzymic activity was determined by continuous measurement of the liberated P_i [17]. The protein concentration was measured according to [18].

2.2. Photoaffinity cross-linking

DiN_3ATP was synthesized as described in [13] by esterification of *N*-4-azido-2-nitrophenyl- β -alanine with 8- N_3ATP . Photoaffinity cross-linking was performed according to [19]. TF_1 (usually 100 μg) was diluted in 200 μl Tris-HCl buffer (100 mM, pH 8.0). After addition of equal concentrations of DiN_3ATP and Mg^{2+} (0.5 mM) the samples were stirred and kept at 37°C during the irradiation (0–60 min). Upon irradiation the sample was applied directly to SDS gel electrophoresis on 7.5% gels [9] after 30 min incubation at 37°C in buffer containing 100 mM Tris-HCl, 1% SDS, 1% 2-mercaptoethanol.

2.3. Hydrolytic cleavage of the cross-link

A labeled sample (100–200 μg TF_1) was subjected to SDS gel electrophoresis. After staining and destaining of the gel the slices containing cross-linked proteins were isolated and incubated in alkaline solution (0.5 M NaHCO_3 , 1% SDS, 0.1 M NaOH) at 37°C for 24 h. After centrifuga-

tion the supernatant was again subjected to SDS gel electrophoresis.

3. RESULTS

3.1. Specific interaction of DiN_3ATP with TF_1

The most important precondition for a successful photoaffinity-labeling experiment is the specific interaction of the photosensitive substrate analog with the protein. This precondition is fulfilled best if the analog is a substrate in the dark or at least a competitive inhibitor for the enzyme.

DiN_3ATP was hydrolyzed by TF_1 in the presence of Mg^{2+} as shown in table 1. The rate of hydrolysis was very low compared with the hydrolysis of Mg^{2+}ATP or Ca^{2+}ATP . The specific binding of DiN_3ATP to TF_1 was also demonstrated by substrate variations in the presence of DiN_3ATP (0.05 and 0.075 mM). DiN_3ATP competitively inhibited the hydrolysis of ATP as shown in fig. 1. The hydrolysis of DiN_3ATP is insignificant and has been neglected. Both results indicate a specific interaction of DiN_3ATP with the hydrolytic site of TF_1 and therefore its suitability as photoaffinity label.

3.2. Light-induced inactivation of TF_1 by DiN_3ATP

Irradiation of TF_1 in the presence of DiN_3ATP led to an inhibition of enzymic activity (fig. 2). This inactivation was not observed if TF_1 was irradiated in the absence of the label (light control). Incubation of TF_1 with DiN_3ATP in the dark only slightly influenced the enzymic activity (dark control).

The addition of ADP or ATP to the sample (TF_1 , DiN_3ATP , Mg^{2+}) prior to irradiation protected TF_1 from inactivation, indicating the nucleotide specificity of the labeling (fig. 3).

Table 1

Hydrolysis of ATP and DiN_3ATP catalyzed by TF_1

Nucleotide	Metal ion	ATPase activity ($\mu\text{mol P}_i/\text{min}$ per mg protein)
0.5 mM ATP	2.5 mM Ca^{2+}	47.7
0.5 mM ATP	0.5 mM Mg^{2+}	25.5
0.5 mM DiN_3ATP	2.5 mM Ca^{2+}	0.0
0.5 mM DiN_3ATP	0.5 mM Mg^{2+}	1.1

AMP, which does not interact in a specific manner with TF_1 (in contrast to ATP or ADP), did not influence the inhibition at all.

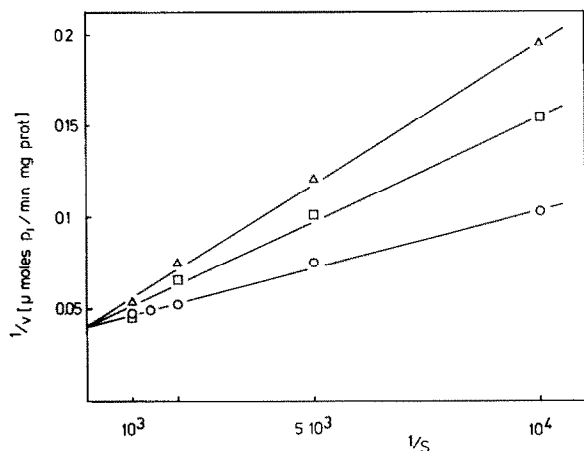


Fig 1 The effect of DiN_3ATP on the hydrolysis of ATP. Plots of $1/v$ vs $1/[Mg \cdot ATP]$ of TF_1 in the absence of DiN_3ATP (○) and in the presence of $Mg \cdot DiN_3ATP$ [0.05 mM (□); 0.075 mM (Δ)]. The ATPase activity was determined at 60°C in 5 ml test solution containing 2 μg TF_1 , 100 mM Tris-HCl (pH 8.0), different concentrations of $Mg \cdot DiN_3ATP$ ($[Mg^{2+}]/[DiN_3ATP] = 1/1$) and $Mg \cdot ATP$ ($[Mg^{2+}]/[ATP] = 1/1$).

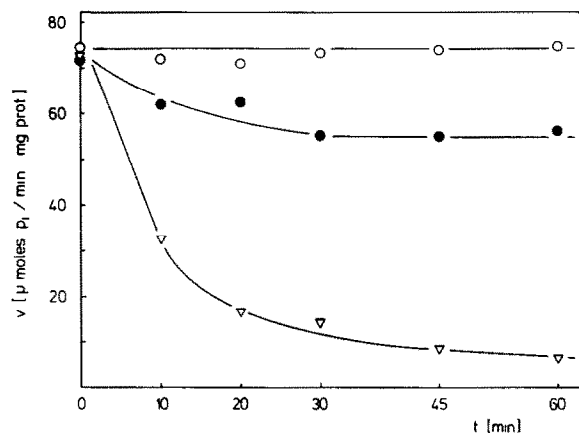


Fig.2. Light-induced inhibition of TF_1 . Irradiation in the presence of 0.5 mM $Mg \cdot DiN_3ATP$ (▽), dark control in the presence of 0.5 mM $Mg \cdot DiN_3ATP$ (●), light control in the absence of DiN_3ATP (○). The enzymic activity was determined at 60°C in 5 ml test solution containing 0.5 μg TF_1 , 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} , and 1 mM ATP.

3.3. Photoaffinity cross-linking of TF_1 subunits with DiN_3ATP

Irradiation of TF_1 in the presence of DiN_3ATP resulted in the formation of higher molecular mass cross-links (>100 kDa) (fig.4, gel b, region 3). Besides the cross-links observed in the region of molecular masses of about 110 kDa, a small amount of even higher molecular mass cross-links could be seen, especially when a greater amount of cross-linked TF_1 was applied to one SDS gel (fig.4,

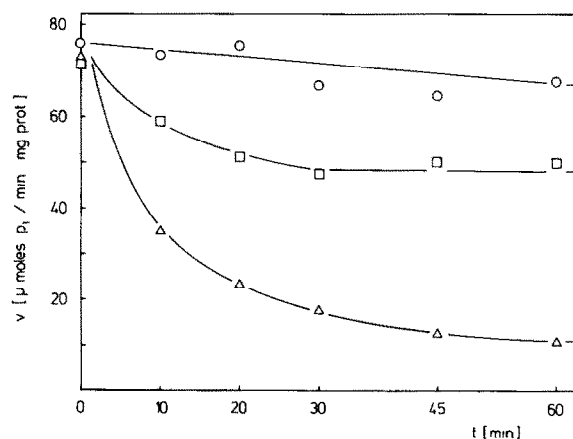


Fig 3 The effect of added Mg nucleotides on the light-induced inhibition of TF_1 by 0.5 mM $Mg \cdot DiN_3ATP$. Additions 1 mM $Mg \cdot AMP$ (Δ), 1 mM $Mg \cdot ADP$ (□), 1 mM $Mg \cdot ATP$ (○). The ATPase activity was determined as described in fig 2.

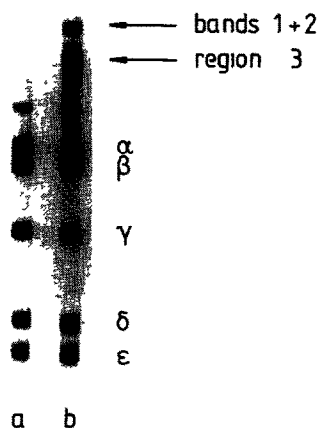


Fig 4 Photoaffinity cross-linking of TF_1 . SDS electrophoresis gels of labeled (cross-linked) TF_1 (a) Native TF_1 (control), (b) TF_1 labeled by 0.5 mM $Mg \cdot DiN_3ATP$.

gel b, bands 1 + 2). All these cross-linked protein bands were not observed with unlabeled TF₁ (fig.4, gel a). The protein band above the α and β bands is a contamination detected in native TF₁ as well. The formation of all these cross-links was nucleotide specific and depended on the presence of Mg²⁺ (fig.5). The weak cross-link bands 1 + 2 near the top of the SDS gel completely disappeared upon addition of ATP or ADP to the sample (enzyme, Mg²⁺, label) prior to irradiation; formation of the main cross-links (region 3) was decreased remarkably (fig.5, gels c + d).

The addition of ATP protected TF₁ more effectively against cross-linking than the addition of ADP. This agrees with the inactivation experiments shown before (fig.3).

In contrast to photoaffinity-labeling of TF₁ with monovalent 8-N₃ATP, photoaffinity cross-linking is partially Mg²⁺ dependent [12]. The weak cross-links (bands 1 + 2) disappeared in the absence of Mg²⁺; the main cross-links (region 3) were formed in a smaller amount (fig.5, gel a).

Indications for the subunit composition of the cross-links were obtained by their hydrolytic cleavage and subsequent SDS gel electrophoresis of the cleavage products. Fig.6 shows that the cross-linked proteins (band 2 and region 3) were split entirely into proteins comigrating with the α and/or β subunit. This indicates that all these cross-links are formed by the major subunits. Due

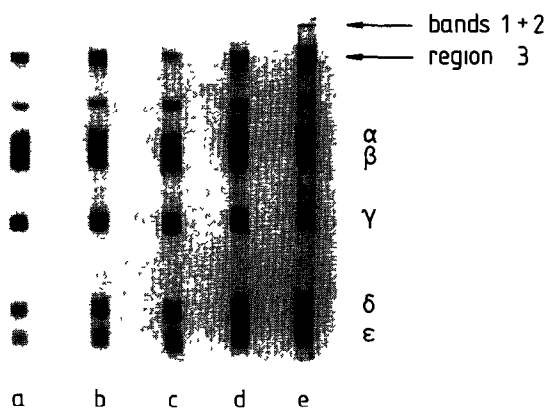


Fig 5 The influence of added effectors on the formation of cross-links. SDS electrophoresis gels of TF₁ labeled by 0.5 mM DiN₃ATP in the presence of (a) 1 mM EDTA, (b) 0.5 mM Mg²⁺, (c) 1.5 mM Mg²⁺ + 1 mM ATP, (d) 1.5 mM Mg²⁺ + 1 mM ADP; (e) 1.5 mM Mg²⁺ + 1 mM AMP

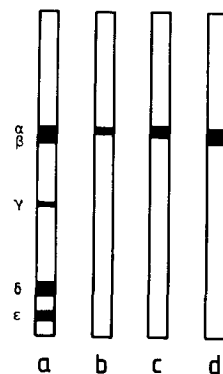


Fig 6. Determination of the cross-link composition. SDS electrophoresis gels. (a) native TF₁ (control); (b) cross-linked protein (band 2) after hydrolytic cleavage, (c) cross-linked protein (region 3) after hydrolytic cleavage, (d) separated α and β subunits after incubation in alkaline solution (identical to b and c)

to the very poor yield of cross-link band 1 it was not possible to determine its composition.

4. DISCUSSION

Our results demonstrate the suitability of DiN₃ATP as a divalent photoaffinity label. It fulfills the criteria for photoaffinity labels [20]. The low hydrolysis rate for DiN₃ATP by TF₁ is analogous to the hydrolysis of DiN₃ATP by F₁ATPase from *M. luteus* [14]. Its behavior is also similar to that of other 2'- and/or 3'-substituted adenosine analogs [21-23]. The high inactivation of ATPase activity, compared with the low amount of cross-link formation, can be explained

in different ways [14]. One plausible possibility is that most of the divalent DiN_3ATP reacts only once with the nucleotide binding site of TF_1 upon irradiation. The second nitrene reacts with water. Only a small part of DiN_3ATP is situated in a position suitable for cross-linking two subunits.

Electron microscopy [24] and cross-linking experiments with divalent group specific reagents [25] have demonstrated the spatial arrangement of $\text{F}_1\text{ATPases}$. An alternating sequence of 3 α and 3 β subunits arranged in two layers was proposed. A top view projection of this arrangement forms the typical hexagonal image of $\text{F}_1\text{ATPases}$ observed in electron microscopy experiments. Such a model possesses α - α , α - β , and β - β interfaces. Recently we were able to demonstrate the localization of a nucleotide binding site at the α - β interface of F_1ATPase from *M. luteus* [14]. The results obtained by photoaffinity cross-linking of TF_1 indicate that there are nucleotide binding sites between the major subunits α and/or β . This is shown by the formation of two-subunit cross-links (region 3). The small amount of even higher molecular mass cross-links suggests the presence of at least two nucleotide binding sites between 3 of the major subunits (band 2).

The compositions α_3 or $\alpha_2\beta$ for this cross-link are most probably based upon its hydrolytic cleavage. The formation of the very weak cross-link band 1 is probably a first indication that more than two nucleotide binding sites (possibly all) are located at interfaces between the major subunits. This has also been suggested for coupling factor 1 from *Escherichia coli* [26] and from chloroplasts [27]. The localization of several nucleotide binding sites between α and/or β subunits is consistent with different models for ATP synthesis/hydrolysis which require strong subunit-subunit interactions [26,28-30]. The reason why trimers or oligomers were detected by cross-linking TF_1 subunits with DiN_3ATP may be the absence of tightly bound nucleotides in TF_1 , which usually occupy tight nucleotide binding sites of $\text{F}_1\text{ATPases}$ [31].

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