Photoaffinity labeling of the coupling factor 1 from the thermophilic bacterum PS3 by 8-azido ATP

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To localize the nucleotide binding sites of the F_1ATPase (TF_1) from the thermophilic bacterium PS3 we have used ^{14}C-labeled 8-azido ATP (8-N_3ATP) as photoaffinity label. 8-N_3ATP is hydrolyzed by the F_1ATPase in the absence of ultraviolet light. Irradiation by ultraviolet light of the enzyme in the presence of 8-N_3ATP results in reduction of ATPase activity and in preferential nucleotide specific labeling of the α subunits (0.8–0.9 mol 8-N_3ATP/TF_1,$\alpha:\beta = 4:1$). Inactivation and labeling do not depend on the presence of Mg^{2+}. Both effects decrease upon addition of various nucleotide di- or triphosphates.

Bacterial F_1ATPase *Thermophilic bacterium PS3* *Photoaffinity labeling* *Catalytic nucleotide binding site* *Noncatalytic nucleotide binding site*

1. INTRODUCTION

Photoaffinity labeling of F_1ATPases from mitochondria, bacteria, and chloroplasts has been successfully applied for the localization of nucleotide binding sites. Three types of photosensitive adenosine nucleotide analogs have been used for this purpose: 3′-arylazidoadenosine nucleotides, 8-azidoadenosine nucleotides, and 2-azidoadenosine nucleotides [1–11]. Owing to the high reactivity of the photoactivated product of 8-N_3ATP, for example, several amino acid residues were shown to be labeled [7]. Labeling of different F_1ATPases has led to diverse results with respect to their subunits. Preferential labeling of the β subunits, an equal labeling of α and β, and preferential labeling of the α subunits have been observed. These discrepancies can be explained by the different labeling conditions employed, or by the different origins of the enzymes. Even the different structures of the photoaffinity labels could be responsible for the diverging results. 8-N_3ATP or 2-N_3ATP, for example, can react immediately at the adenine binding site, whereas 3′-arylazidoadenosine nucleotides may label the enzymes at locales more distant from the adenine binding site. Here we report on a specific labeling of F_1-ATPase from the thermophilic bacterium PS3 by irradiation in the presence of 8-N_3ATP. In contrast to other F_1s, the TF_1 preparation does not contain bound nucleotide and divalent metal ions, and is stable against many nonspecific chemical reagents. Thus TF_1 is better suited to analyze ATP-Mg binding activity.

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Abbreviations: TF_1, coupling factor 1 (F_1ATPase) from the thermophilic bacterium PS3; 8-N_3ATP, 8-azido ATP, 8-azidoadenosine 5′-triphosphate; 3′-arylazido-β-alanyl-ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}adenosine 5′-triphosphate; 3′-arylazido-β-alanyl-8-azido ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-8-azidoadenosine 5′-triphosphate; 8-N_3AMP, 8-azidoadenosine 5′-monophosphate; 2-N_3ATP, 2-azidoadenosine 5′-triphosphate

2. MATERIALS AND METHODS

2.1. *Preparation of F_1ATPase from the thermophilic bacterium PS3*

TF_1 was prepared from plasma membranes of

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PS3 as in [12]. After exhaustive dialysis of the purified enzyme against distilled water it was stored as a lyophilized powder at −20°C. Lyophilized TF_1 was dissolved in Tris–HCl buffer (100 mM, pH 8.0) just before a series of experiments.

The enzymic activity was determined by continuous measurement of the liberated P_i [13]. Usually 1–2 μg TF_1 were dissolved in 10 ml Tris–HCl buffer (100 mM, pH 8.0). Immediately after the addition of the nucleotide (ATP, or 8-N_3ATP) and Me(II) ions (Mg^{2+}, or Ca^{2+}) the formation of P_i was studied for 10 min at 60°C. The protein concentration was determined as in [14].

2.2. *Photoaffinity labeling*

8-N_3ATP was prepared from ATP via 8-BrATP as in [15]. Photoaffinity labeling was performed in a simple apparatus according to [16]. The samples were irradiated with a Minerallight handlamp UVSL 25 at 'long wave'. The energy fluence rate at the position of the sample was 4 W/m^2. TF_1 (usually 50 μg) was diluted in 250 μl Tris–HCl buffer (100 mM, pH 8.0). Equal concentrations of 8-N_3ATP and Mg^{2+} (1 mM) were added. The samples were stirred vigorously and kept at 37°C during the irradiation (60 min). After irradiation the labeled protein was precipitated with trichloroacetic acid at 4°C. The precipitate was applied to SDS gel electrophoresis on 7.5% gels as in [9] with the following modification: the samples were incubated for 30 min at 37°C before application to the gel.

Radioactivity was determined in a Packard Tricarb liquid scintillation counter model 3380 according to [17].

3. RESULTS

3.1. *8-N_3ATP as substrate for TF_1*

A precondition for the useful application of a substrate analog for affinity/photoaffinity labeling is the specific interaction of this analog with the investigated protein. One criterion for a suitable photoaffinity label is to be a substrate or at least a competitive inhibitor for the enzyme in the dark. Table 1 shows the hydrolysis of different Me(II) nucleotides at 60°C. 8-N_3ATP is hydrolyzed by TF_1 in the presence of Mg^{2+} or Ca^{2+}. The hydrolytic cleavage of 8-N_3ATP demonstrates specific interactions of the nucleotide with the hydrolytic site of the enzyme. Additional interactions are not excluded by this result.

Table 1

Hydrolysis of ATP and 8-N_3ATP in the presence of Ca^{2+} or Mg^{2+} catalyzed by TF_1

Nucleotide	Metal ion	ATPase activity (μmol P_i/min per mg protein)
1 mM ATP	5 mM Ca^{2+}	65.9
1 mM ATP	1 mM Mg^{2+}	27.3
1 mM 8-N_3ATP	5 mM Ca^{2+}	1.1
1 mM 8-N_3ATP	1 mM Mg^{2+}	3.0

3.2. *Light-induced inactivation of TF_1 by 8-N_3ATP*

Irradiation of TF_1 in the presence of 8-N_3ATP and Mg^{2+} results in a drastic reduction of ATPase activity (fig.1). The same degree of inactivation is observed after photoaffinity labeling in the presence of EDTA. Irradiation of the enzyme in the absence of the label, or incubation of TF_1 with 8-N_3ATP in the dark, do not influence the enzymic activity. The nucleotide specificity of the labeling could be demonstrated by competition experiments. ATP and ADP compete with 8-N_3ATP

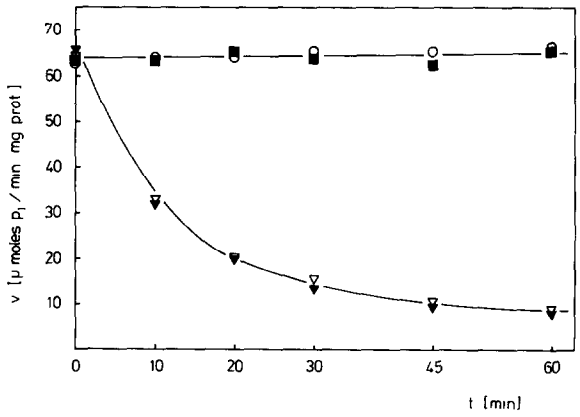

Fig.1. Light-induced inhibition of TF_1. Irradiation in the presence of 1 mM 8-N_3ATP + 1 mM Mg^{2+} (▽); 1 mM 8-N_3ATP + 1 mM EDTA (▼); light control in the absence of 8-N_3ATP (○); dark control in the presence of 1 mM 8-N_3ATP + 1 mM Mg^{2+} (■). *t*, irradiation time; *v*, ATPase activity.

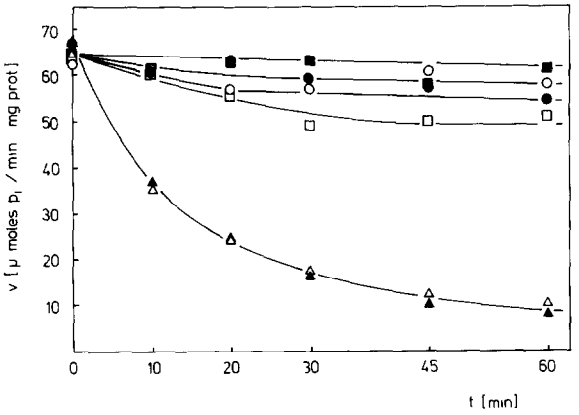

Fig.2. The effect of added nucleotides on the light-induced inhibition of TF_1 by 1 mM 8-N_3ATP. Addition of 1 mM AMP + 2 mM Mg^{2+} (△); 1 mM AMP + 2 mM EDTA (▲); 1 mM ADP + 2 mM Mg^{2+} (□); 1 mM ADP + 2 mM EDTA (■); 1 mM ATP + 2 mM Mg^{2+} (○); 1 mM ATP + 2 mM EDTA (●). *t*, irradiation time; *v*, ATPase activity.

for the nucleotide binding site. Therefore, addition of ATP or ADP before the irradiation results in a protection of TF_1 against inactivation (fig.2). The protection of enzymic activity by ADP or ATP is also observed in the absence of Mg^{2+}. Addition of AMP, however, does not influence the degree of inactivation by photoaffinity labeling.

3.3. *Covalent labeling of TF_1 by irradiation in the presence of 8-N_3ATP*

Irradiation of TF_1 in the presence of 8-N_3ATP and Mg^{2+} results in a preferential labeling of the α subunits (table 2). The incorporation of the nucleotide into the α subunit corresponds directly with the reduction of enzymic activity, indicating that 100% inhibition is reached after covalent binding of a single nucleotide to the α subunits of TF_1. Irradiation of TF_1 in the presence of 8-N_3ATP and EDTA results in quite the same amount of covalently bound label. This demonstrates the independence of the labeling on the presence of Mg^{2+}. Addition of ATP, ADP, ITP, or GTP in presence or absence of Mg^{2+}, protects TF_1 against the incorporation of 8-N_3ATP. The labeling rate is not influenced by addition of AMP. Irradiation of TF_1 in presence of 8-N_3AMP which does not interact specifically with the enzyme results in a labeling rate nearly identical with that of the protection experiments. In all these experiments the incorporation of the label into the α subunit agrees directly with the reduction of enzymic activity, although about 20% of the total label was found in the β subunit.

Table 2

Influence of various effectors on photoaffinity labeling of the α and β subunits of TF_1 by 8-N_3[^{14}C]ATP and 8-N_3[^{14}C]AMP

Label	Effectors	Nucleotide/TF_1 on		Inactivation (%)
		α	β	
1 mM 8-N_3ATP	1 mM Mg^{2+}	0.64	0.17	67
	1 mM EDTA	0.68	0.14	67
	1 mM AMP, 2 mM Mg^{2+}	0.72	0.23	65
	1 mM AMP, 2 mM EDTA	0.61	0.15	71
	1 mM ADP, 2 mM Mg^{2+}	0.25	0.12	22
	1 mM ADP, 2 mM EDTA	0.14	0.09	13
	1 mM ATP, 2 mM Mg^{2+}	0.24	0.08	21
	1 mM ATP, 2 mM EDTA	0.16	0.10	19
	1 mM ITP, 2 mM Mg^{2+}	0.46	0.15	45
	1 mM ITP, 2 mM EDTA	0.40	0.11	39
	1 mM GTP, 2 mM Mg^{2+}	0.38	0.11	34
	1 mM GTP, 2 mM EDTA	0.24	0.09	15
1 mM 8-N_3AMP	1 mM Mg^{2+}	0.23	0.06	26

Photoaffinity labeling was performed as described in section 2. At the position of γ, δ and ϵ no significant radioactivity could be observed

4. DISCUSSION

The results fulfill the criteria for a suitable photoaffinity label as defined for 3'-arylazido-β-alanyl-ATP [4].

(i) 8-N_3ATP is a substrate of TF_1.
(ii) 8-N_3ATP is covalently bound to TF_1 by irradiation.
(iii) Covalent binding results in inactivation of ATPase activity.
(iv) Other nucleotides (ATP, ADP, ITP, GTP) which interact specifically with TF_1 protect against photoinactivation.

A comparison of these results with those obtained for the photoaffinity labeling of F_1ATPase from *Micrococcus luteus* shows two important differences. Irradiation of the *M. luteus* enzyme in the presence of 8-N_3ATP results in a nucleotide specific labeling of the β subunits. This labeling depends strongly on the presence of Mg^{2+} [9]. Photoaffinity labeling of TF_1, however, results in an Mg^{2+}-independent labeling of the α subunits.

We want to discuss two explanations for the diverging results with the two bacterial F_1ATPases.

(i) It has been shown that mitochondrial F_1ATPase possesses 6 nucleotide binding sites: 3 catalytic binding sites on the β subunits and 3 noncatalytic tight nucleotide binding sites on the α subunits [18]. F_1ATPase from *M. luteus* contains 2.5–3.0 tightly bound nucleotides [19]. They are likely to occupy the noncatalytic nucleotide binding sites on α. Therefore, photoaffinity labeling of this enzyme takes place at the catalytic sites on β – the hydrolysis of nucleotides is Mg^{2+} dependent. TF_1, however, does not contain tightly bound nucleotides [20,21]. In this case an Mg^{2+}-independent labeling of the noncatalytic binding sites may occur. The catalytic sites of TF_1 are situated at the β subunits as well [20]. A consequence of this hypothesis is that covalent labeling of the noncatalytic nucleotide binding sites on α results in the inactivation of TF_1.
(ii) It is also possible that the nucleotide binding sites are situated at the interface between α and β. In this case 8-N_3ATP can label either the α or the β subunit depending on the exact orientation of the label at the binding site between the two major subunits. Strong evidence for the position of nucleotide binding sites between the α and the β subunits is provided by photoaffinity cross-linking with the bifunctional 3'-arylazido-β-alanyl-8-azido ATP [22]. Irradiation of F_1ATPase from *M. luteus* [23], of the oligomycin-sensitive ATPase from beef heart mitochondria [24] and of TF_1 (unpublished) in the presence of this ATP analog results in a nucleotide specific formation of α-β cross-links. This hypothesis, however, cannot explain the different Mg^{2+} dependence.

These two possible explanations are not mutually exclusive. It is conceivable that all the different results obtained for photoaffinity labeling of F_1ATPases [1–11] are due to different combinations of both possibilities.

The 1:1:1 ADP·Mg·TF_1 complex [20] was shown to produce the ATP·Mg·TF_1 complex by the method in [25], and the actual substrate for TF_1 was shown to be the Δ,β,γ-bidentate Mg·ATP complex [26]. The ^{31}P-NMR analyses of ADP·TF_1 also revealed the tight binding of 1 ADP per TF_1 at the catalytic site (unpublished). These data suggest that the labeled site may be located at the α-β interface of TF_1.

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