Photoaffinity cross-linking of F₁ATPase from the thermophilic bacterium PS3 by 3'-arylazido-β-alanyl-8-azido ATP

Hans-Jochen Schäfer, Gabriele Rathgeber, Klaus Dose, Y. Masafumi and Y. Kagawa

Institut für Biochemie, Johannes Gutenberg-Universität, J. J. -Becher-Weg 30, D-6500 Mainz, FRG and *Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Tochigi-ken 329-04, Japan

Received 24 April 1985, revised version received 13 May 1985

To study the localization of the nucleotide binding sites of coupling factor 1 (TF₁) from the thermophilic bacterium PS3 we used the bifunctional (cross-linking) 3'-arylazido-β-alanyl-8-azido ATP (DiN₃ATP) for photoaffinity labeling. DiN₃ATP is hydrolyzed by TF₁ in the absence of ultraviolet light. Irradiation (UV light) of TF₁ in the presence of DiN₃ATP 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.

1. INTRODUCTION

Photoaffinity labeling of different F₁ATPases 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 Mg²⁺, 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 DiN₃ATP [13].

If the nucleotide binding site is localized at or near the interface, the two nitrene groups formed upon irradiation of DiN₃ATP 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 DiN₃ATP 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 F₁ATPase from Micrococcus luteus by DiN₃ATP led to the formation of an α-β cross-link [14].

Abbreviations. TF₁, coupling factor 1 (F₁ATPase) from the thermophilic bacterium PS3, DiN₃ATP, 3'-arylazido-β-alanyl-8-azido ATP, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]proponyl]-8-azidoadenosine 5'-triphosphate; 8-N₃ATP, 8-azido ATP, 8-azidoadenosine 5'-triphosphate.
Upon irradiation of oligomycin-sensitive ATPase from beef heart mitochondria in the presence of DiN3ATP 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 DiN3ATP. The reason why TFI was used is (i) it does not contain firmly bound nucleotides and Mg2+, (ii) it is very stable against nonspecific chemical treatment and (iii) it is reconstitutable from its subunits after denaturation with SDS [16].

2. MATERIALS AND METHODS

2.1. Preparation of F1ATPase from the thermophilic bacterium PS3

TFI was prepared from plasma membranes of PS3 as described [12,16]. The absence of bound nucleotides and Mg2+ in TFI used was confirmed. The enzymic activity was determined by continuous measurement of the liberated P, [17] The protein concentration was measured according to [18].

2.2. Photoaffinity cross-linking

DiN3ATP was synthesized as described in [13] by esterification of N4-azido-2-nitrophenyl-β-alanine with 8-N3ATP. Photoaffinity cross-linking was performed according to [19]. TFI (usually 100 μg) was diluted in 200 μl Tris-HCl buffer (100 mM, pH 8.0). After addition of equal concentrations of DiN3ATP and Mg2+ (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 TFI) 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 NaHCO3, 1% SDS, 0.1 M NaOH) at 37°C for 24 h. After centrifugation the supernatant was again subjected to SDS gel electrophoresis.

3. RESULTS

3.1. Specific interaction of DiN3ATP with TFI

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. DiN3ATP was hydrolyzed by TFI in the presence of Mg2+ as shown in table 1. The rate of hydrolysis was very low compared with the hydrolysis of Mg2+ATP or Ca2+ATP. The specific binding of DiN3ATP to TFI was also demonstrated by substrate variations in the presence of DiN3ATP (0.05 and 0.075 mM). DiN3ATP competitively inhibited the hydrolysis of ATP as shown in fig.1. The hydrolysis of DiN3ATP is insignificant and has been neglected. Both results indicate a specific interaction of DiN3ATP with the hydrolytic site of TFI and therefore its suitability as photoaffinity label.

3.2. Light-induced inactivation of TFI by DiN3ATP

Irradiation of TFI in the presence of DiN3ATP led to an inhibition of enzymic activity (fig.2). This inactivation was not observed if TFI was irradiated in the absence of the label (light control). Incubation of TFI with DiN3ATP in the dark only slightly influenced the enzymic activity (dark control).

The addition of ADP or ATP to the sample (TFI, DiN3ATP, Mg2+) prior to irradiation protected TFI from inactivation, indicating the nucleotide specificity of the labeling (fig.3).

Table 1

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Metal ion</th>
<th>ATPase activity (μmol P, /min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM ATP</td>
<td>2.5 mM Ca2+</td>
<td>47.7</td>
</tr>
<tr>
<td>0.5 mM ATP</td>
<td>0.5 mM Mg2+</td>
<td>25.5</td>
</tr>
<tr>
<td>0.5 mM DiN3ATP</td>
<td>2.5 mM Ca2+</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5 mM DiN3ATP</td>
<td>0.5 mM Mg2+</td>
<td>11.1</td>
</tr>
</tbody>
</table>
AMP, which does not interact in a specific manner with TF₁ (in contrast to ATP or ADP), did not influence the inhibition at all.

Fig. 1 The effect of DiN₃ATP on the hydrolysis of ATP. Plots of $1/v$ vs $1/[Mg\cdot ATP]$ of TF₁ in the absence of DiN₃ATP (○) and in the presence of Mg DiN₃ATP [0.05 mM (○); 0.075 mM (△)]. The ATPase activity was determined at 60°C in 5 ml test solution containing 2 μg TF₁, 100 mM Tris-HCl (pH 8.0), different concentrations of Mg·DiN₃ATP ([Mg²⁺]/[DiN₃ATP] = 1/1) and Mg·ATP ([Mg²⁺]/[ATP] = 1/1).

Fig. 2. Light-induced inhibition of TF₁. Irradiation in the presence of 0.5 mM Mg·DiN₃ATP (△), dark control in the presence of 0.5 mM Mg·DiN₃ATP (○), light control in the absence of DiN₃ATP (○). The enzymatic activity was determined at 60°C in 5 ml test solution containing 0.5 μg TF₁, 100 mM Tris-HCl (pH 8.0), 5 mM Ca²⁺, and 1 mM ATP.

3.3. Photoaffinity cross-linking of TF₁ subunits with DiN₃ATP

Irradiation of TF₁ in the presence of DiN₃ATP 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 TF₁ was applied to one SDS gel (fig. 4, gel b, region 3).

Fig. 3 The effect of added Mg nucleotides on the light-induced inhibition of TF₁ by 0.5 mM Mg DiN₃ATP. Additions 1 mM Mg AMP (△), 1 mM Mg·ADP (○), 1 mM Mg·ATP (○). The ATPase activity was determined as described in fig. 2.

Fig. 4. Photoaffinity cross-linking of TF₁ SDS electrophoresis gels of labeled (cross-linked) TF₁ (a) Native TF₁ (control), (b) TF₁ labeled by 0.5 mM Mg·DiN₃ATP.
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 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 DiN₂ATP reacts only once with the nucleotide binding site of TFr upon irradiation. The second nitrene reacts with water. Only a small part of DiN₂ATP 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 F₁ATPases. 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 F₁ATPases 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 F₁ATPase from M. luteus [14]. The results obtained by photoaffinity cross-linking of TFr 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 α₃ or α₂-β 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 TFr subunits with DiN₂ATP may be the absence of tightly bound nucleotides in TFr₁, which usually occupy tight nucleotide binding sites of F₁ATPases [31].

ACKNOWLEDGEMENTS

The authors thank M. Mittelmann-Sicurella, Universität Mainz, for editing the manuscript and E.-M. Rückwardt, Universität Mainz, for preparation of the manuscript. This work was supported by Deutsche Forschungsgemeinschaft Grant Sch 344/1-2 to H.-J. S.

REFERENCES


279