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PHOTOAFFINITY CROSS-LINKING OF OLIGOMYCIN-SENSITIVE ATPase FROM BEEF HEART MITOCHONDRIA BY 3'-ARYLAZIDO-8-AZIDO ATP

Hans-Jochen Schäfer[+], Luise Mainka[°], Gabriele Rathgeber[+], and Guido Zimmer[°]

[+]Institut für Biochemie der Universität, J.J.Becher-Weg 30, D-6500 Mainz, West Germany

[°]Gustav-Embden-Zentrum der Biologischen Chemie, Universität Frankfurt, Theodor-Stern-Kai 7, D-6000 Frankfurt am Main, West Germany

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SUMMARY: Photoaffinity cross-linking of the oligomycin-sensitive ATPase from beef heart mitochondria by 3'-arylazido--8-azido ATP results in a nucleotide specific formation of a cross-link between α and/or ß subunits. Moreover a nucleotide independent decrease of the heterogeneous 29-31 kd protein band is observed. This decrease can be reduced by addition of 2,4-dinitrophenol or 2-azido-4-nitrophenol.

The bifunctional photosensitive ATP analog 3'-arylazido--8-azido ATP (DiN_3ATP) is an agent suitable for studying the neighborhood of nucleotide binding sites [1]. Recently we have shown that irradiation of bacterial F_1 ATPase in the presence of DiN_3ATP results in specific formation of a cross-linked protein [2]. This cross-link is formed by one α and one ß subunit indicating that the nucleotide binding site at the ß subunit [3] is located near the α subunit.

Oligomycin-sensitive ATPases prepared by different laboratories differ in the composition of the F_0 part. Especially, a protein band of 29-31 kd is missing in some preparations [4,5], and has been claimed to be essential for oligomycin-sensitivity in others [6,7]. Discussion about its requirement for ATP-P_i exchange is controversial [5,8]. Recently, a parti-

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culate ATPase-ATP-synth(et)ase that exhibits an extremely high ATP-P_i exchange activity was also found to contain both a very high amount of lipid (1.14 mg phospholipids/mg protein) and of 29-31 kd protein [8]. In this investigation we have applied DiN_3ATP to the oligomycin-sensitive ATPase from beef heart mitochondria in order to study the arrangement of the subunits.

MATERIALS AND METHODS

Isolation of beef heart mitochondria:
Beef heart mitochondria were isolated according to the method of Smith [9]. Preparation of submitochondrial particles followed the procedure of Racker [10]. Protein was estimated by the method of Lowry et al. [11].

Preparation of oligomycin-sensitive ATPase complex (OS-ATPase):
OS-ATPase was prepared according to the method described by Serrano et al. [12]. The sucrose gradient centrifugation was left out. The final pellets of OS-ATPase were suspended in 10 mM Tris-sulfate, 0.5 mM EDTA, 1.0 mM $MgSO_4$ and 50 mM sucrose pH 7.5, and stored at -80 °C. ATPase activity in solution was determined by continuous measurement of the liberated inorganic phosphate as described by Arnold et al. [13]. Usually 3-4 µg protein were dissolved in 5 ml Tris-HCl buffer [100 mM, pH 8.0]. Immediately after addition of MgATP the formation of inorganic phosphate was assayed for 5 min.

Substances:
8-Azidoadenosine 5'-triphosphate ($8\text{-}N_3ATP$) was synthesized as described by Schäfer et al. [14]. 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl} 8-azidoadenosine 5'-triphosphate (3'-arylazido-8-azido ATP, DiN_3ATP) was synthesized according to Schäfer et al. [1] by esterification of N-4-azido-2-nitrophenyl-ß-alanine with $8\text{-}N_3ATP$. 2-Azido-4-nitrophenol was kindly provided by Dr. W.G. Hanstein, Universität Bochum.

Photoaffinity labeling and photoaffinity cross-linking:
Photoaffinity labeling and photoaffinity cross-linking was performed in an apparatus described by Schäfer et al. [15]. The samples were irradiated with a Mineralight handlamp UVSL 25 at position 'long wave'. The energy fluence rate at the position of the sample (4 cm from the light source) was 4 W per m^2. Usually 2 mg protein were diluted in 400 µl Tris-sulfate buffer [10 mM, pH 7.5] containing EDTA [0.5 mM], $MgSO_4$ [1 mM], and sucrose [50 mM]. After the addition of DiN_3ATP [0.2, 0.5 mM] the samples were stirred vigorously and kept at 25 °C during the irradiation (30 min). In some experiments effectors (ATP, ADP, AMP, $8\text{-}N_3ATP$, 2,4-dinitrophenol, or 2-azido-4-nitrophenol) were added prior to the irradiation. The concentration of these effectors was 1 mM in all cases.

Slab gel electrophoresis in polyacrylamide:
The gradient slab gel electrophoresis was carried out using 5-15 % gels. The electrode buffer was 50 mM Tris, 0.38 mM glycine, 0.1 % sodium dodecylsulfate, 2 mM EDTA pH 8.8. About 70-110 µg protein was applied to the gels. The gels were electrophoresed at 30 mA constant current for about 2 h. Fixation and

staining were carried out with 0.5 % (w/v) Serva Blau-R in methanol:acetic acid:H_2O [25:10:65]. Destaining was done with the same solvent.

RESULTS

Irradiation of OS-ATPase with ultraviolet light (λ >300 nm) in the presence of DiN_3ATP and Mg^{2+} ions results in an inhibition (>95 %) of ATPase activity (Fig.1). Incubation of the enzyme with DiN_3ATP in the dark or irradiation of the enzyme in the absence of the photoaffinity label had only small influence on the enzymic activity (<20-30 % inactivation). SDS gel electrophoresis of the labeled enzyme shows a weak additional protein band in the region of higher molecular weights (> 100 kd) and a strong decrease of the 29-31 kd protein band (Fig.2c+3b).

These effects are not observed in the following control experiments: a. irradiation of OS-ATPase in the absence of Di-

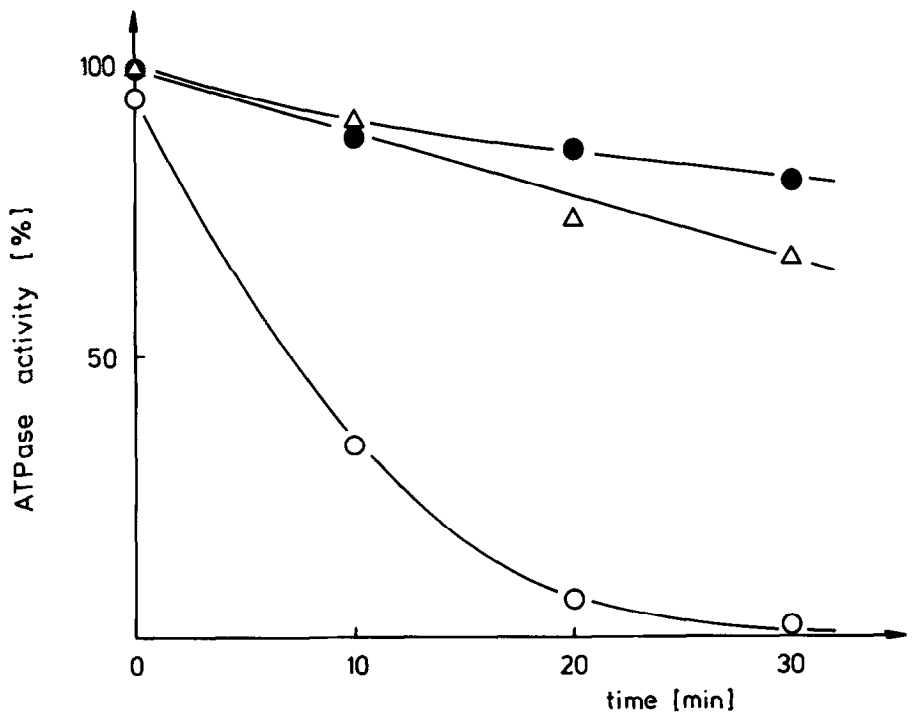

Fig.1 Light-induced inhibition of OS-ATPase by 0.5 mM Mg·DiN_3-ATP (○), light control in the absence of DiN_3ATP (△), dark control in the presence of 0.5 mM Mg·DiN_3ATP (●).

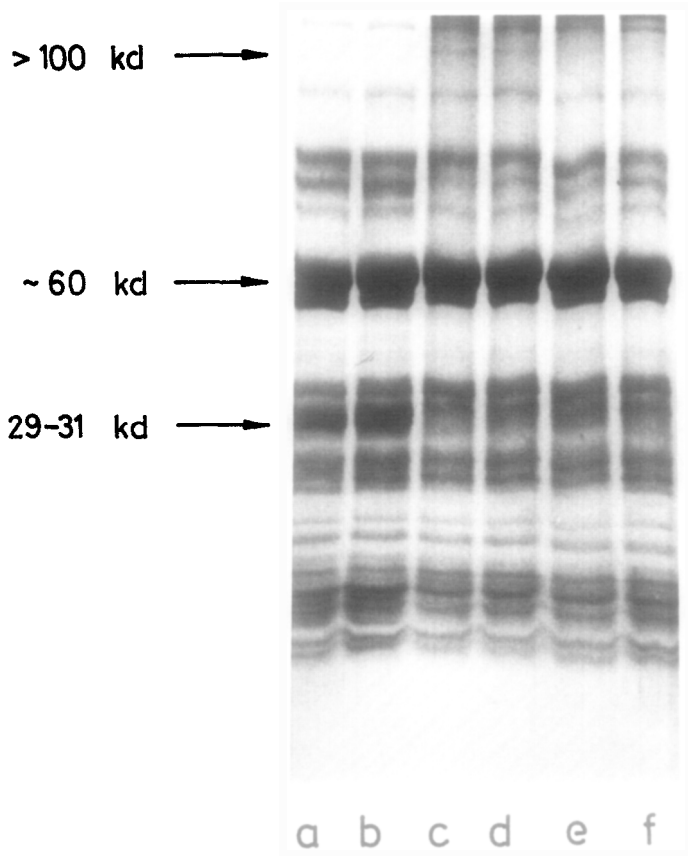

Fig.2 SDS electrophoresis gels of labeled OS-ATPase:
a. OS-ATPase irradiated in the absence of DiN_3ATP (light control).
b. OS-ATPase incubated with 0.2 mM $Mg \cdot DiN_3ATP$ (dark control).
c. OS-ATPase labeled by 0.2 mM $Mg \cdot DiN_3ATP$.
d.-f. OS-ATPase labeled by 0.2 mM $Mg \cdot DiN_3ATP$ in the presence of 1 mM Mg·AMP (d), 1 mM Mg·ADP (e), or 1 mM Mg·ATP (f).

N_3ATP (Fig.2a), b. incubation of OS-ATPase with DiN_3ATP in the dark (Fig.2b), c. irradiation of OS-ATPase in the presence of the monofunctional $8\text{-}N_3ATP$ (Fig.3f).

Addition of ADP or ATP prior to the irradiation prevents nearly completely the formation of the protein band in the higher molecular weight region (Fig.2e+f). AMP does not show this effect (Fig.2d). The decrease of the 29-31 kd protein band is not significantly influenced by addition of these nucleotides (Fig.2d-f). In contrast to this effect the addition

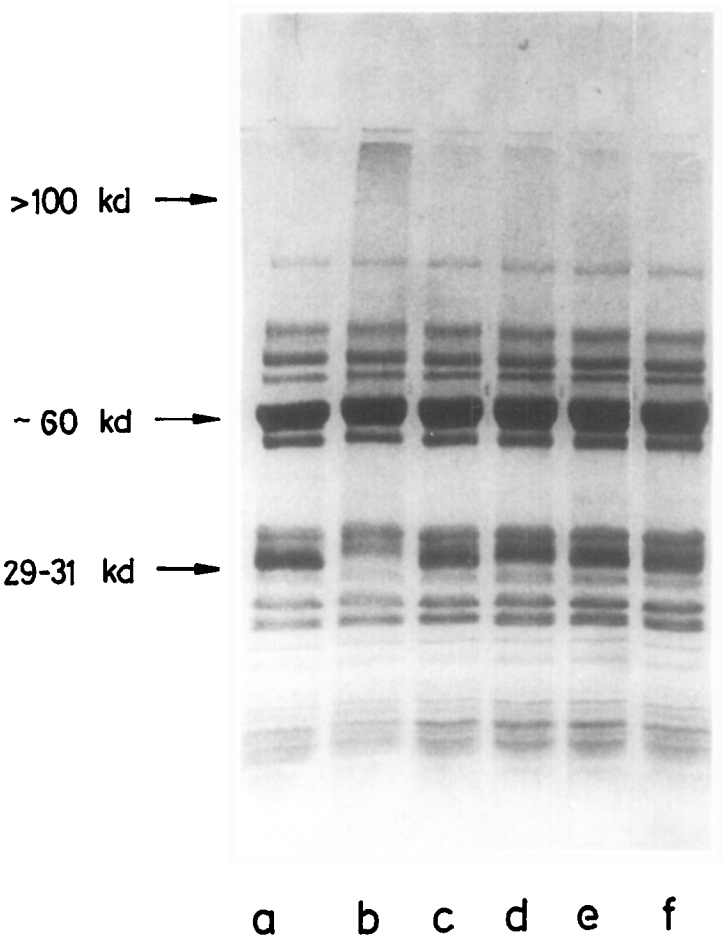

Fig.3 SDS electrophoresis gels of labeled OS-ATPase:
a. OS-ATPase (control).
b. OS-ATPase labeled by 0.2 mM Mg·DiN$_3$ATP.
c. OS-ATPase labeled by 0.2 mM Mg·DiN$_3$ATP in the presence of 1 mM 2,4-dinitrophenol.
d. OS-ATPase labeled by 0.2 mM Mg·DiN$_3$ATP in the presence of 1 mM 2-azido-4-nitrophenol.
e. OS-ATPase labeled by 1 mM 2-azido-4-nitrophenol.
f. OS-ATPase labeled by 1 mM Mg·8-N$_3$ATP.

of phenols like 2,4-dinitrophenol, or 2-azido-4-nitrophenol prior to the labeling procedure results in a protection of the 29-31 kd protein against cross-linking (Fig.3c+d).

DISCUSSION

The additional protein band at > 100 kd that is observed after irradiation of the OS-ATPase in the presence of DiN$_3$-ATP is supposedly analogous to the α-β cross-link obtained

by photoaffinity cross-linking of the bacterial F_1 ATPase [2]. The decrease in the formation of this cross-link by addition of ATP or ADP prior to the irradiation indicates that a specific nucleotide binding site is involved in this cross-link.

In spite of the decrease of the heterogenous 29-31 kd protein band after photoaffinity-cross-linking no additional protein band of higher molecular weight with a comparable intensity could be observed. Therefore, we conclude that cross-linking of this (these) protein(s) results in an approx. 60 kd cross-link. This additional protein band, however, was not observed due to the high amount of protein (α+ß subunit) in this molecular weight region. Similar results are obtained when using diepoxybutane for cross-linking [16]. A further indication for an approx. 60 kd cross-link results from experiments with bromobimanes [17,18]. Labeling of OS-ATPase with monobromobimane leads to a fluorescent band at 29-31 kd. Using the cross-linking dibromobimane an additional fluorescent band in the region of the α and the ß subunit appears at the expense of the 29-31 kd fluorescence [18].

The decrease of the 29-31 kd protein is nearly independent of the addition of ATP, ADP and AMP indicating that probably no nucleotide binding site participates in the cross-linking of this (these) protein(s).

The prevention of 29-31 kd protein cross-linking in the presence of aromatic compounds like 2,4-dinitrophenol or 2-azido-4-nitrophenol suggests that the cross-linked protein(s) are identical with some or all of the membrane-integrated protein(s) labeled by 2-azido-4-nitrophenol [19-21] and by photoreactive 2-azido-4-nitrophenyl-phospholipids [7].

Nucleotide-translocase [22,23], phosphate carrier [24,25] and uncoupler binding protein(s) [19-21] belong to this molecular weight range. Due to this complexity the molecular composition of the cross-link(s) has not yet been revealed.

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