PHOTOAFFINITY LABELLING OF A LOW-AFFINITY NUCLEOTIDE BINDING SITE ON THE β-SUBUNIT OF YEAST MITOCHONDRIAL F_1-ATPase

Roland GREGORY, Diether RECKTENWALD, Benno HESS, Hans-Jochen SCHÄFER+, Peter SCHEURICH+ and Klaus DOSE+

Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, D-4600 Dortmund 1 and *+Institut für Biochemie der Joh. Gutenberg Universität, Joh. Joachim Becher Weg 30, D-6500, Mainz, FRG*

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1. Introduction

Mitochondrial ATP synthase from yeast is an oligomeric enzyme consisting of a hydrophobic membrane part (F_0) and a hydrophilic part (F_1). F_1 is composed of 5 different polypeptide chains (α to ε) [1].

Low- and high-affinity nucleotide di- and triphosphate binding sites have been characterized on the enzyme from various sources by different kinetic techniques [2,3]. Alternatively, binding sites have been located on specific subunits by photoaffinity labelling [4–8]. Recently, the development of a fluorescent photoaffinity label (8-azido-1,N^6-etheno-ATP) was reported [9], which might allow the identification of a binding site with respect to the hydrolytic or synthetic function of F_1. We have therefore investigated the interaction of this compound with yeast F_1. It will be shown that the label interacts with a low-affinity nucleotide binding site, and that upon photolabelling it is bound to the β-subunit.

2. Materials and methods

The enzyme was prepared according to [1] and stored as a suspension in 70% saturated $(NH_4)_2SO_4$.

Abbreviations: ε-N_3-ADP, 8-azido-1,N^6-etheno-ADP; ε-N_3-ATP, 8-azido-1,N^6-etheno-ATP; HEPPS K, 4-[2-hydroxyethyl]-1-piperazine propane sulphonate, K^+ salt; NEM, *N*-ethyl maleimide

To transfer the enzyme to the appropriate buffer the $(NH_4)_2SO_4$ suspension was spun down at 100 000 × *g* for 60 min and the pellet dissolved in 50 mM HEPPS K, 2 mM EDTA (pH 7.9) medium. The resulting solution was desalted by passage through a Sephadex G-25 column according to [10].

Reagents for electrophoresis were obtained from either Serva (Heidelberg) or Merck (Darmstadt). All solvents and bulk chemicals were reagent grade. ε-N_3-ATP was synthesized according to [9]. An equimolar mixture of ε-N_3-ADP and phosphate was prepared by reacting ε-N_3-ATP with a catalytic amount of F_1 in the above medium for 2 min at 25°C.

The enzyme activity was determined according to [11]. The steady state kinetics were measured according to [1]. The dependence of the enzyme stability on the ε-N_3-ATP concentration was measured according to [3].

Photoinactivation experiments were carried out by irradiating up to 3 samples simultaneously as given in table 1 with a Mineral Light lamp UVSL 25 in position 'long wave'. The energy flux rate at the position of the samples was 4000 W/m^2. The enzyme solution containing the ε-N_3-ATP was stirred vigorously during the irradiation at 25°C. Before electrophoresis, non-covalently bound ε-N_3-ATP was removed by centrifuging the sample through Sephadex G-25 equilibrated with the medium [10].

SDS–polyacrylamide gel electrophoresis, staining and destaining, were performed according to [12]. Fluorescent bands on the gel were located by visual inspection of the gels under ultraviolet light and

their position was marked by insertion of a piece of wire. After fixing and staining, the position of the stained bands could be compared with that of the wire.

3. Results

The subunit showing significant photolabelling by ϵ-N_3-ATP was found to be the β-subunit of yeast F_1. As shown in fig.1, the relative mobilities of both the fluorescent label and the β-subunit were identical and ~0.32. Table 1 also shows that the labelling was accompanied by a loss of the ATPase activity.

In order to correlate the photolabelled binding site with known nucleotide binding sites (of which there are at least two types, high- and low-affinity), the influence of ATP on the inactivation by possible competition with the analogue for the site, was studied. Table 1 shows that indeed, in the presence of 2 mM ATP during the illumination a marked stabilization against photoinactivation was observed. Nevertheless a high degree of fluorescent label was bound to the β-subunit. This is to be expected for the case of a competition between the binding of ATP and the irreversible reaction of the photolabelled compound. The influence of filtering of ultraviolet light by the adenine nucleotide moiety is negligible as shown by the low protection in the presence of 2 mM AMP. Other nucleotide triphosphates such as d-ATP, ϵ-ATP and ITP, as an indicator of low-affinity sites, also protected against the photoinactivation (results not shown). These experiments clearly show that ϵ-N_3-ATP competes with other nucleotide triphosphates for nucleotide binding sites of low specificity.

To provide additional information that ϵ-N_3-ATP binds to low-affinity sites the steady state kinetics of ϵ-N_3-ATP hydrolysis were analyzed. A K_m of 0.2 mM was found compared with 0.04 mM for ATP [1], also indicating that the hydrolysis occurs at a low-affinity site.

Since the long-term stabilization of the ATPase activity by various ligands is a good criterion for the binding to high-affinity sites [3], the effect of the photolabel on the F_1 stability was tested. ATP and ITP were added as controls. Figure 2 shows the experiment where the ATPase activity in solutions of

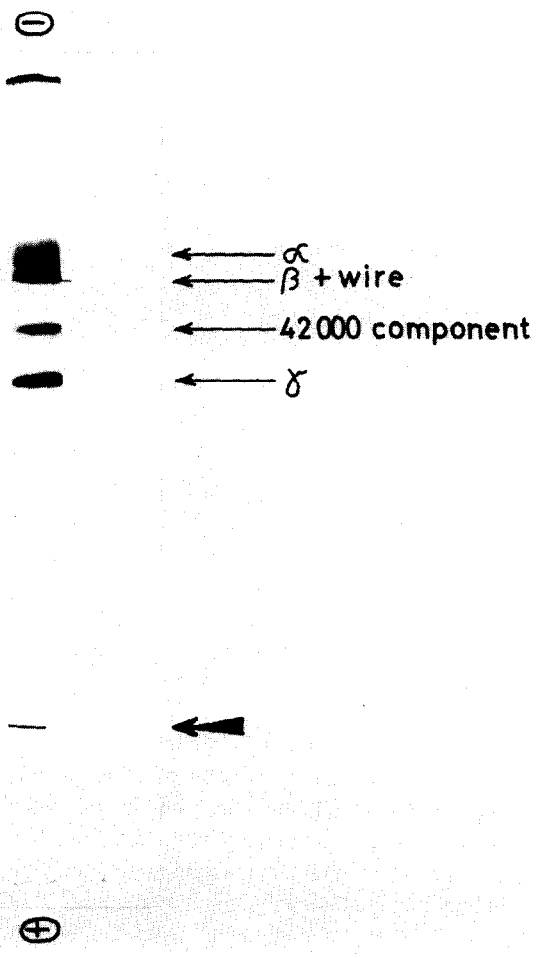

Fig.1. SDS–polyacrylamide gel electrophoresis of F_1 after photoaffinity-labelling with ϵ-N_3-ATP. The fluorescent band was marked with a wire before staining with Coomassie blue. The lower arrow indicates the position of the marker dye. The sample contained ~30 μg F_1. A high concentration of protein is necessary for detection of the fluorescent band and this alters the relative staining intensity of the various bands. For the subunit structure of yeast F_1 refer to [1]. For details see section 2.

Table 1
Percentage of the ATPase activity remaining after 15 min illumination in various additions to the medium at 25°C (1 mg F_1/ml)

1 mM ϵ-N_3-ATP, 2 mM Mg^{2+}	8
1 mM ϵ-N_3-ADP, 1 mM P_i, 2 mM Mg^{2+}	8
1 mM ϵ-N_3-ATP, 2 mM EDTA	11
1 mM ϵ-N_3-ATP, 2 mM AMP, 2 mM Mg^{2+}	20
1 mM ϵ-N_3-ATP, 2 mM ATP, 2 mM Mg^{2+}	60

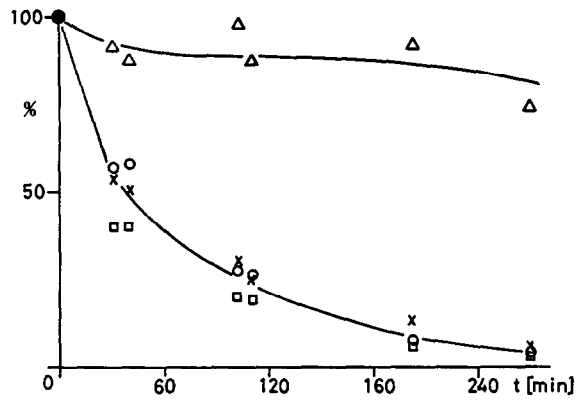

Fig.2. Comparison of the capacities of different nucleotide triphosphates to stabilize F_1 during long-term incubation in the dark. F_1 (5 U or ~25 μg/ml) was incubated in the presence of: (△) 0.9 mM ATP; (□) 0.9 mM ITP; (○) 0.9 mM ϵ-N_3-ATP; (X) no addition. Buffer was 50 mM HEPPS K, 2 mM EDTA (pH 7.9).

different nucleotide triphosphates (including the photolabel) containing originally 5 U/ml of enzyme is plotted against time, demonstrating that ATP protects the enzyme against inactivation (triangles). In contrast, the results with ϵ-N_3-ATP (circles) are not significantly different from those with ITP (squares) or in the absence of nucleotide triphosphates (crosses). We further found that a mixture of 0.5 mM ϵ-N_3-ATP and 0.2 mM ATP stabilized similarly to ATP (not shown here). This demonstrates that the observed loss of ATPase activity in the presence of analogue (fig.2) is not caused by a chemical reaction of the analogue with F_1 in the dark. Thus, both steady state kinetics and the stabilization experiments indicate that ϵ-N_3-ATP cannot bind to the high-affinity nucleotide sites on F_1.

Because of the hydrolytic activity of F_1, the above experiments do not answer the question whether the photoaffinity label is bound as the triphosphate, or after hydrolysis, as the diphosphate. Therefore, the photolabelling and photoinactivation experiments were repeated using an equimolar mixture of ϵ-N_3-ADP and phosphate instead of ϵ-N_3-ATP. Table 1 shows that the loss of ATPase activity was comparable to that observed with the corresponding triphosphate. From this we conclude that the ϵ-N_3-ATP is first split and then reacts with the enzyme.

4. Discussion

For yeast F_1-ATPase, ϵ-N_3-ATP meets the criteria of a photoaffinity label as defined for arylazido-β-alanyl-ATP [8], namely:

(i) It is a substrate of F_1 ATPase;
(ii) It is covalently bound after illumination;
(iii) Covalent binding results in inactivation of the ATPase activity;
(iv) Other nucleotide triphosphates protect against photoinactivation.

Since the criteria of photoaffinity-labelling are met and, in addition, our results show that the criteria can be applied to the low-affinity site, our results clearly show that we have specifically labelled a functionally-defined nucleotide binding site. Presently, quantitative information about the binding could not be obtained, because the fluorescence quantum yield of the enzyme–label complex in SDS–gels is not known.

A comparison of our data with those obtained with F_1 from other sources shows that the assignment of a low affinity binding site to the β-subunit in yeast fits observations obtained with beef heart F_1, which has comparable properties for its β and α subunit, namely, the aurovertin binding site on β [13,14], and the distribution of sulfhydryl groups over the α and not the β subunit in both cases ([15], R.G., B.H., unpublished). Indeed, a photoaffinity label comparable to ours, 8-azido-ATP, binds also to the β-subunit of beef heart F_1 [5]. The question whether the photolabelled low affinity site is involved in ATP synthesis cannot be answered. However, independent experiments [2,3] suggest that at least the low-affinity sites with low specificity are not involved in ATP- or energy-dependent conformational changes in F_1. From this we suggest that the site labelled by ϵ-N_3-ATP might not be involved in ATP synthesis.

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