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SPECIFIC PHOTOLABELLING OF BEEF-HEART MITOCHONDRIAL ATPase BY 8-AZIDO-ATP

R. J. WAGENVOORD, I. VAN DER KRAAN and A. KEMP

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

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SUMMARY

1. 8-Azido-ATP is a suitable photoaffinity label for beef-heart mitochondrial ATPase (F_1).

2. 8-Azido-ATP is hydrolysed slowly by F_1 in the dark. Photolysis at 350 nm in the presence of F_1 leads to inhibition of the ATPase activity. The presence of ATP during illumination prevents the inhibition. Illumination of F_1 in the absence of 8-azido-ATP causes no inhibition.

3. Added Mg^{2+} is not necessary for the binding of the 8-azido-ATP to F_1.

4. 8-Azido-ATP binds specifically to the β subunits of F_1.

5. The ATPase activity is completely inhibited when 2 mol of 8-azido-ATP are bound per mol F_1.

INTRODUCTION

Photoaffinity substrate analogues have been successfully used for labelling substrate-binding sites in enzymes. Knowles [1] has defined the criteria that such a label should possess. It should be acted upon the enzyme like the natural substrate and bind specifically to the enzyme, it should be photolysed on irradiation with light of a wavelength that does not damage the protein, its photolysis product should be reactive with all groups present in proteins and the photolysis product should not be rapidly converted into a less reactive compound, thereby losing its capacity to label the binding site of the protein.

Azido compounds have been widely used for this purpose. On irradiation with near ultraviolet light (about 350 nm), to which proteins are little sensitive, these compounds photolyse with formation of the highly reactive nitrene [1–3] and nitrogen. Intramolecular rearrangements are minimized when the azido groups and therefore the nitrene group are coupled to a ring system. 8-Azido-ADP has been used as a photoaffinity inhibitor of the adenine nucleotide translocator [4] and 8-azido-[β-^{32}]ATP has been used to label the ($Na^+ + K^+$)-ATPase of red blood cells [5].

Abbreviation: F_1, mitochondrial ATPase.

We have synthesized 8-azido-ATP, tritium labelled in the adenine ring, and used it photoaffinitely to label beef-heart mitochondrial ATPase, F_1, the terminal enzyme in oxidative phosphorylation [6, 7]. 8-Azido-ATP is hydrolysed by F_1 (in the dark) indicating that it binds specifically to the ATP-binding site. On irradiation in the presence of 8-azido-ATP the ATPase activity of F_1 is irreversibly inhibited. The inhibition is strongly diminished by the presence of ATP during irradiation, suggesting competition between 8-azido-ATP and ATP for the ATP-binding site(s). We conclude that 8-azido-ATP fulfils all criteria for a suitable photoaffinity label for the mitochondrial ATPase.

METHODS AND MATERIALS

Synthesis of 8-azido-ATP. Bromination of AMP, according to Muneyama et al. [8], gave 8-bromo-AMP in 89 % yield. 8-Azido-AMP was obtained in 58 % yield by treating 8-bromo-AMP, dissolved in dimethylformamide, with a 2-fold excess of tributylammonium azide and heating the mixture overnight at 75 °C (cf. refs. 5 and 8). 8-Azido-ATP was obtained in 40 % yield by phosphorylation of 8-azido-AMP according to Michelson [9]. The impure 8-azido-ATP was purified on a DEAE-cellulose column (4.5×40 cm) with a linear gradient (100–400 mM) of triethylammonium bicarbonate (pH 7.5) in a volume of 4 l (cf. ref. 5). The appropriate fractions were combined and evaporated in vacuum at 0–5 °C. The 8-azido-ATP was precipitated as the lithium salt [9] in ethanol/acetone (1:1, v/v), and dried above P_2O_5 in vacuum. The purified compound had an absorption maximum at 282 nm (cf. refs. 5 and 10) and photolysed at 350 nm. The changes in the absorption spectrum during photolysis were very similar to those reported for 8-azido-ADP [10].

8-Azido-[2-^{3}H]ATP was synthesized from [2-^{3}H]AMP.

F_1. Beef-heart mitochondrial ATPase F_1 was prepared according to Knowles and Penefsky [11]. The ATPase activity (defined as μmol ATP hydrolysed per min per mg protein at 30 °C) was measured spectroscopically at 340 nm in a Zeiss spectrophotometer (PMQ II) at 30 °C. The cuvette contained 83 mM sucrose, 6 mM $MgCl_2$, 33 mM Tris/acetate, 16.7 μM NADH, about 1 μg/ml F_1 and, as an ATP-regenerating system, 0.5 mM potassium phosphoenolpyruvate, 3.6 units (μmol/min) lactate dehydrogenase and 1.5 units (μmol/min) pyruvate kinase. The final pH was 8.0. The reaction was started by addition of 5 mM ATP. Protein concentrations of F_1 solutions were measured according to Lowry et al. [12], with bovine serum albumin ($A_{279\,nm}^{1\%} = 6.67$) as standard.

Light-induced inhibition and labelling of F_1 by 8-azido-ATP. A small dish, diameter 25 mm, containing F_1 dissolved in 1–2 ml of 250 mM sucrose, 10 mM Tris/acetate buffer (pH 7.5), 2 mM EDTA and 8-azido-ATP, was illuminated with ultraviolet light at 350 nm (6 cm from a CAMAG, Type TL 900, 8 W lamp) at room temperature. After the illumination the ATPase activity was measured and in case of a labelling experiment with 8-azido-[2-^{3}H]ATP the free nucleotides were removed by precipitating the F_1 in 50 % saturated $(NH_4)_2SO_4$ and after 10 min at 0 °C centrifuging in an Eppendorf centrifuge (type 3200). The supernatant was carefully discarded and the F_1 pellet was dissolved in 250 mM sucrose, 10 mM Tris/acetate buffer (pH 7.5) and 2 mM EDTA. Residual unbound nucleotides were removed first by adding activated carbon, removing the carbon after 20 min by centrifugation and

passing the clear supernatant through a Dowex 1-X8 column (0.5×2 cm). The eluate was used for the determination of the amount of label bound to F_1 and to investigate which subunit(s) of F_1 were labelled.

Determination of radioactivity. The radioactivity of the samples was determined in a Nuclear Chicago liquid scintillation counter type MARK I. As scintillation liquid was used a mixture of three parts of toluene and one part Triton X-100, in which were dissolved per l 3 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyl oxazolyl-2) benzene. Per sample 10 ml of the scintillation liquid were added.

Polyacrylamide gel electrophoresis. Electrophoresis on 12 % polyacrylamide gels in the presence of dodecyl sulphate was carried out according to Weber et al. [13], using stacking gels as described by Maurer [14]. The gel tubes had a diameter of 0.5 cm and a length of 11 cm. Before applying to the gels 18 μl 20 % (w/v) sodium dodecyl sulphate, 90 μl glycerol, 36 μl 0.02% bromphenol blue, 4.72 mg Na_2CO_3, 16.7 μg iodoacetic acid, 100 μl pure mercaptoethanol and water to 1.0 ml were added to the protein solution, and the mixture was heated for 4 min at 100 °C. The electrophoresis was carried out at 20 °C. A current of 1.5 mA was used to stack the protein (30–45 min), after which it was increased to 3 mA and the gel run for an additional 2.5 h. The electrode buffer contained 50 mM Tris/glycine buffer (pH 8.8–8.9) and the upper reservoir contained in addition 0.03 % (w/v) sodium dodecyl sulphate.

After electrophoresis the gels were stained with 0.25 % Coomassie Blue in acetic acid/methanol/water (1:4:5, by vol.). After staining for 2–16 h, they were destained in acetic acid/methanol/water (1:4:5, by vol.). The gels were stored in 5 % acetic acid.

Scanning and slicing the gels. The gels were scanned at 500–550 nm, depending on the intensity of the staining, using a Zeiss (Z.K. 4) gel scanner. To determine the radioactivity incorporated in different protein bands they were, after the scanning, frozen in solid CO_2 and cut in a gel slicer (Mickle) in slices of 2 mm. The slices were extracted by shaking for 2 h at 48 °C, or overnight at room temperature, in 90 % (by vol.) aqueous protosol. The radioactivity was measured after addition of 10 ml scintillation liquid.

Materials. Dowex 1-X8 (100–200 mesh) was obtained from Bio Rad Laboratories, DEAE-cellulose medium mesh from Sigma Chemical Co., Triton X-100 from B.D.H. Chemicals Ltd., protosol from New England Nuclear, [2-^{3}H]AMP from the Radiochemical Centre, Amersham.

RESULTS

Hydrolysis of 8-azido-ATP by F_1

The hydrolysis of 8-azido-ATP is catalysed by F_1 with Michaelis-Menten kinetics. The K_m is 2.6 mM and the V is 3.0 μmol/min per mg protein, compared with 0.5 mM and 100 μmol/min per mg protein, respectively, measured for ATP in parallel experiments. Since 8-azido-ADP has a lower affinity than ADP for pyruvate kinase, 10 times more pyruvate kinase was used in the assay for the 8-azido-ATPase activity than in the ATPase assay.

Light-induced inhibition of the ATPase activity of F_1 by 8-azido-ATP

Fig. 1A shows that on irradiation with ultraviolet light 8-azido-ATP inhibits

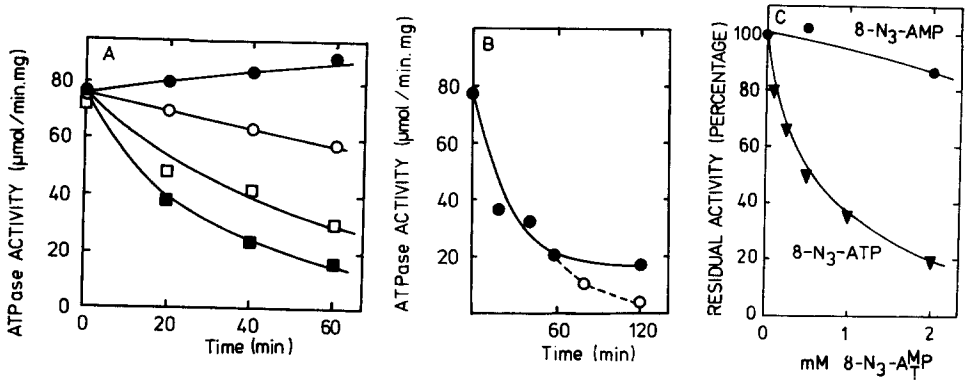

Fig. 1. Light-induced inhibition of the ATPase activity of F_1 by 8-azido-ATP. The reaction medium contained 250 mM sucrose, 10 mM Tris/acetate buffer (pH 7.5), 2 mM EDTA and different concentrations of 8-azido-ATP or 8-azido-AMP. (A) Effect of 0.25 mM (○), 1.0 mM (□) and 2.0 mM (■) 8-azido-ATP. The control without 8-azido-ATP (●) is also shown. (B) Effect of 4 mM 8-azido-ATP (●). The points indicated by ○ were obtained by precipitating the F_1 with $(NH_4)_2SO_4$ after 60 min irradiation with 4 mM 8-azido-ATP, and continuing the irradiation after resuspending in a fresh medium containing 4 mM 8-azido-ATP. (C) Effect of different concentrations of 8-azido-ATP concentrations (▼) and 8-azido-AMP (●) after 1 h illumination.

the ATPase activity of F_1. The inhibition depends on the time of irradiation and concentrations of 8-azido-ATP. After some time the rate of the inhibition slows down, probably due to photolysis of 8-azido-ATP to 8-hydroxamino-ATP [10]. When further 8-azido-ATP is added, the inhibition continues virtually to completion (Fig. 1B).

Fig. 1C shows that irradiation in presence of 8-azido-AMP causes only a slight inhibition of the ATPase activity of F_1, indicating that 8-azido-AMP does not bind to F_1.

Fig. 2 shows that ATP protects against the light-induced inactivation, presumably by competing with 8-azido-ATP for non-covalent binding to F_1. Since AMP has only a slight effect the protection by ATP is not due to light absorption by the adenine ring.

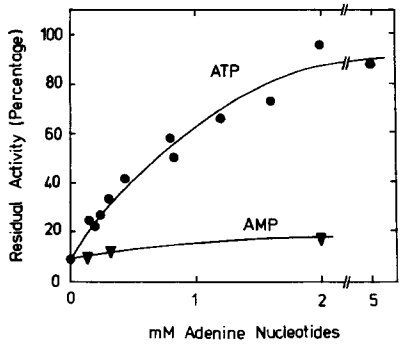

Fig. 2. Effect of ATP (●) and AMP (▼) on light-induced inhibition of the ATPase activity of F_1 by 8-azido-ATP. 2 mM 8-azido-ATP, 60 min irradiation. The reaction medium was the same as in Fig. 1.

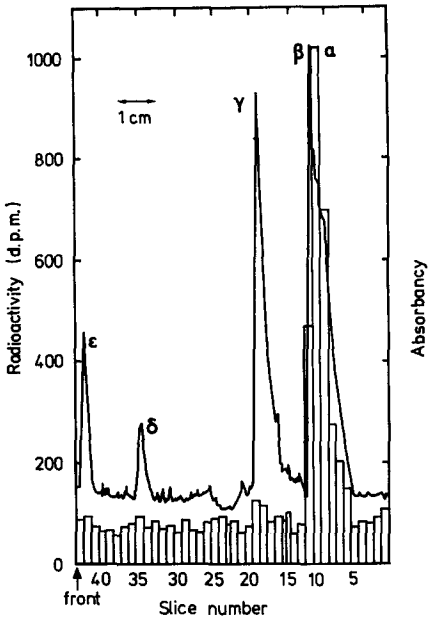

Fig. 3. Polyacrylamide gel electrophoresis in presence of dodecyl sulphate of F_1 labelled by 8-azido-[2-^{3}H]ATP. 50 μg F_1 labelled with 8-azido-[2-^{3}H]ATP (16 000 dpm/nmol) were applied to the gel. The continuous line shows the scanning at 500 nm after Coomassie-blue staining. The radioactivity of the slices is given in the bars.

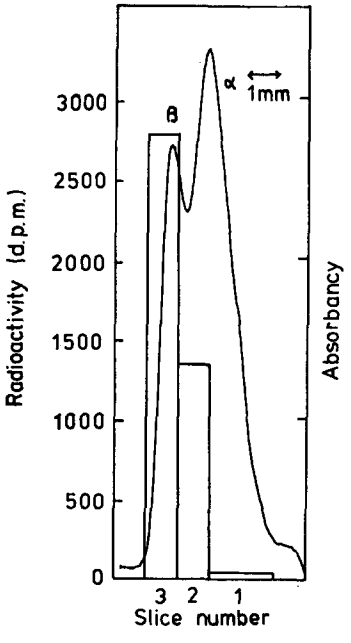

Fig. 4. Polyacrylamide gel electrophoresis in presence of dodecyl sulphate of F_1 labelled by 8-azido-[2-^{3}H]ATP. 7.5 μg F_1 labelled with 8-azido-[2-^{3}H]ATP (80 000 dpm/nmol) were applied to the gel. Other details are the same as in Fig. 3.

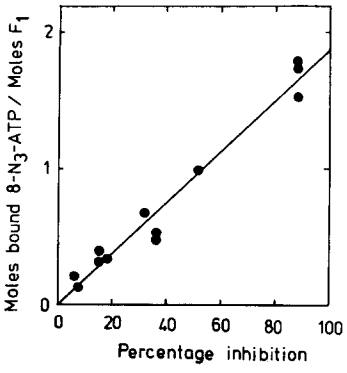

Fig. 5. Bound 8-azido-ATP (tritium labelled) as a function of the light-induced inhibition of the ATPase activity of F_1. By varying 8-azido-ATP concentrations and illumination time, different inhibition percentages were obtained. The bound 8-azido-[2-^{3}H]ATP was determined as described in Methods and Materials.

Since both the light-induced inhibition of the ATPase and the protection by ATP take place in the absence of added Mg^{2+}, it is clear that both 8-azido-ATP and ATP can bind to F_1 without added Mg^{2+}.

Radioactive labelling of the subunits of F_1

In order to determine which subunits of F_1 are labelled, photoaffinity experiments were carried out with 8-azido-[2-^{3}H]ATP, and the photolysed mixture, after removal of free nucleotides, was examined by gel electrophoresis as described in Methods and Materials. The scan of the gel, given in Fig. 3, shows the normal pattern for F_1, except that the α and β subunits are not separated because of the high amount of protein applied to the gels (50 μg). The other subunits γ, δ and ε are well separated. Only the α–β region of the gel is radioactively labelled. The constant level around 80 dpm found for the rest of the gel is equal to the background which was not subtracted.

By applying much less (7.5 μg) F_1 to the gel an almost complete separation of the α and β subunits was obtained. Fig. 4 gives the scan of the α–β region of the gel, which shows clearly two bands, only one of which (the β band) is radioactively labelled.

Fig. 5 shows that the amount of bound 8-azido-[2-^{3}H]ATP per mol F_1 (based on a molecular weight of 360 000 [15]) is proportional to the percentage light-induced inhibition of the ATPase activity, and by extrapolation to 100 % inhibition it can be concluded that 2 mol 8-azido-ATP are bound per mol F_1. Thus there are two binding sites for 8-azido-ATP per mol F_1, and in view of the competition between ATP and 8-azido-ATP for the binding sites, we may conclude that there are at least two binding sites for added ATP per mol F_1.

DISCUSSION

From these experiments we conclude that 8-azido-ATP fulfils the criteria for a suitable photoaffinity label for F_1. It is hydrolysed by F_1 and can be photolysed

under conditions in which the protein is not damaged. It binds specifically to the β subunit of F_1 and maximally 2 mol are bound per mol F_1.

Preliminary experiments with the ATPase complex isolated from submitochondrial particles indicate that the F_1 in the complex is also photoaffinity labelled with 8-azido-ATP.

The fact that the two 8-azido-ATP-binding sites are present in the β subunit fraction makes it rather likely that 2 β subunits are present in F_1. On the basis of *N*-ethylmaleimide binding, Senior [16] has proposed that 2 α, 2 γ and 2 ε subunits are present in a molecule F_1. Since the β and δ subunits do not contain reactive -SH groups, he was unable to determine the stoichiometry of these subunits. Combining our results with these of Senior [16] we propose $\alpha_2 \beta_2 \gamma_2 \delta_x \varepsilon_2$ for the subunit structure of F_1, where x is probably 1 or 2.

Our conclusion that the ATP-binding site is on the β subunits is consistent with the finding of Ferguson et al. [17] that reaction of a single tyrosine in F_1 with 4-chloro-7-nitrobenzofurazan leads to complete inhibition of the ATPase activity and that the reactive tyrosine is close to a lysine nitrogen atom on the side chain of the β subunits.

The presence of two ATP-binding sites is also of interest in relation to the two-site mechanism proposed by Kayalar et al. [18].

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