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The use of 8-azido-ATP and 8-azido-ADP as photoaffinity labels of the ATP synthase in submitochondrial particles: evidence for a mechanism of ATP hydrolysis involving two independent catalytic sites?

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8-Azido-ATP is a substrate for the ATP synthase in submitochondrial particles with a V_{max} equal to 6% of the V_{max} with ATP. The K_m values for 8-azido-ATP are similar to those for ATP. ATP synthase in submitochondrial particles can bind maximally 2 mol 8-N-ATP or 8-N-ADP per mole and the inhibition of ATP hydrolysis by covalently bound N-ATP or N-ADP is proportional to the saturation of the enzyme with inhibitor, similar to the results obtained with isolated F_1. Both 8-N-ATP and 8-N-ADP are bound mainly to the β subunits and at all levels of saturation the distribution of the label is 77% to the β and 23% to the α subunits. It is proposed that the binding of 8-azido-AXP itself is mainly to the β subunit, but that part of the nitreno radicals formed during excitation with light reacts with an amino acid of the α subunit, due to the location of the binding site at an interface between a β and an α subunit. Partial saturation with 8-N-ATP, under conditions that the concentration of 8-azido-ATP during the incubation is intermediate between the low and high K_m values, does not abolish the apparent negative cooperativity of ATP hydrolysis. It is concluded that this apparent cooperativity is not due to the presence of two different catalytic sites, nor to a cooperativity between the two catalytic sites, but to interaction between the catalytic sites and regulatory sites.

Introduction

8-Azido-ATP and 8-azido-ADP have been shown to be very useful tools to investigate the properties and function of the adenine nucleotide-binding sites in isolated mitochondrial ATPase [1,2]. 8-Azido-ATP is hydrolyzed by isolated F_1 with a K_m 3-times higher than that of ATP and a V_{max} 15-times lower. The binding of both analogues after illumination is noncooperative and with 2 moles analogue bound per mol F_1 the enzyme is fully inhibited. 8-azido-ATP (in the presence of EDTA) binds preferentially to the β subunits. 8-Azido-ADP (in the presence of Mg^{2+}) binds preferentially to the α subunits and hinders the subsequent binding of 8-azido-ATP to the β subunits [1,2]. In this paper these studies have been extended to a more intact system, the ATP synthase in phosphorylating submitochondrial particles. It will be shown that also in sub-

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Abbreviations: 8-N-ATP, 8-nitreno-adenosine-5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; S-13, 2,5-dichloro-3-tertiarybutyl-4′-nitrosalicylanilide; SDS, sodium dodecyl sulphate; AXP, ADP or ATP.

mitochondrial particles binding of 8-N-ADP or 8-N-ATP is non-cooperative and that a difference with isolated F_1 can be demonstrated in the availability of regulatory sites for 8-azido-ADP during the resting state.

Materials and Methods

Materials

All common chemicals were of analytical grade. Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, NADH, ATP and ADP were purchased from Boehringer, Mannheim. Antimycin was obtained from Sigma. S13 was a gift from Dr. P. Hamm, Monsanto Company, St. Louis, MO, U.S.A. 8-Azido-ADP and 8-azido-ATP were synthesized by A.F. Hartog according to Schäfer et al. [4] in our laboratory. For the synthesis of the tritiated compounds the starting material was [2-^{3}H]ATP purchased from the Radio Chemical Centre, Amersham. Protosol was delivered by New England Nuclear and the scintillation liquid used was Scintillator 299 from Packard.

Methods

Biological preparations

Heavy bovine heart mitochondria were isolated essentially according to Smith's procedure 3 [5] with the modification that 10 mM Tris (pH 7.8) was replaced by 12 mM $KH_2PO_4 \cdot KOH$ (pH 7.4) and that the concentration of succinate was raised to 10 mM. The mitochondria were stored at −100°C at about 80 mg/ml. Submitochondrial particles (MgMnATP) were prepared from this suspension essentially according to Hansen and Smith [6] with the modification that the Tris-HCl buffer was replaced by 10 mM Hepes-KOH (pH 7.5). Soluble F_1 was isolated as described by Knowles and Penefsky [7].

The protein concentration in soluble F_1 preparations was determined as described by Lowry et al. [8]. As a standard procedure for determining the protein concentration of a suspension of submitochondrial particles, the Lowry method was also used, calibrated with the biuret procedure of Cleland and Slater [9]. The calibration showed that the results of the Lowry method overestimated, in a reproducible way, the protein content by 10%.

Photolabelling of submitochondrial particles was carried out in a buffer containing 250 mM sucrose, 10 mM Hepes-KOH and, in the case of 8-azido-ATP, 5 mM EDTA. The final pH was 7.5. With 8-azido-ADP, EDTA was replaced by 2 mM Mg^{2+}. The concentrations of 8-azido-AXP were as indicated in the legends. The protein content was 10 mg/ml or less. The suspension was kept in shallow glass vials thermostatically controlled at about 2°C and at a distance of about 5 cm from a CAMAG TL-900/U ultraviolet lamp. After a few minutes of preincubation the suspension was illuminated with near ultraviolet light with a wavelength of 366 nm for a certain time-period as specified in the legends. After this period the particles were centrifuged through a Sephadex G-50 coarse column [10], equilibrated with the illumination buffer, to remove unbound photolysed product. A sample was taken from the eluate and to the remainder new 8-azido-AXP was added and the illumination procedure was repeated.

Usually the illumination was repeated several times. In a parallel experiment control particles were incubated under the same conditions, ATP replacing 8-azido-ATP and ADP replacing 8-azido-ADP. ATPase activities were calculated relative to the activity of these control samples.

The ATPase activity of the control samples was found to increase (2-3)-fold during the whole procedure. Most likely, this effect is due to removal of the inhibitor protein from the F_1 on the columns. To avoid this problem a batch of inhibitor-depleted particles was prepared as described by Racker and Horstmann [11] with the modification that MgMnATP particles were used instead of A-particles. The specific ATPase activity (in the presence of uncoupler) was indeed increased by a factor of 3 due to this procedure, but coupling was almost lost: the P/O ratio with succinate as substrate decreased from 0.7 to 0.05 (data not shown). Submitochondrial particles treated in this way no longer showed an increase in ATPase activity on centrifugation through a column or after incubation with trypsin (data not shown). Such inhibitor-depleted submitochondrial particles were used for all radioactive photolabelling experiments.

ATPase activity was routinely measured in a medium containing 83 mM sucrose, 33 mM Tris-HCl buffer (pH 8.0), 10 mM $KHCO_3$, 6 mM $MgCl_2$, 5 mM ATP, 0.5 mM phospho*enol*pyru-

vate, 250 μM NADH, 3 μM rotenone, 2.5 U pyruvate kinase/ml, 2 U acetate dehydrogenase/ml and 500 nM of the uncoupler S-13,

The reaction rate was measured at 30°C by the disappearence of NADH using a Zeiss M 4 Q III spectrophotometer.

For K_m determinations the ATPase medium contained: 250 mM sucrose, 10 mM KP_i, 0.1 mM EDTA, 1 mM Mg^{2+}, 1 mg/ml bovine serum albumin, 1 mM phospho*enol*pyruvate, 250 μM NADH, 2.5 U pyruvate kinase/ml, 2 U lactate dehydrogenase/ml and 3 μM rotenone at pH 7.5. Substrate was added as Mg-ATP or Mg-8-azido-ATP.

ATP synthesis was measured by incubating submitochondrial particles at 30°C, in an 1.6 ml oxygraph vessel equipped with a Clark oxygen electrode. The incubation medium contained 250 mM sucrose, 4 mM $MgCl_2$, 0.1 mM EDTA, 10 mM potassium phosphate, 4 mM AMP, 10 mM Hepes, 1 mM NADH, 20 mM glucose and 4.2 units of hexokinase/1.6 ml (final pH 7.5). The ADP was added to concentrations as indicated in the figure. The reaction was started by adding the submitochondrial particles to the medium. When almost all the oxygen was consumed, 1 ml of the reaction mixture was added to 300 μl of ice-cold 14% perchloric acid containing 40 mM EDTA. After centrifugation for 2 min at 15000 × *g*, 1 ml of the supernatant was neutralized with 6 M KOH/0.3 M Mops. The formed $KClO_4$ was removed by centrifugation for 2 min at 15000 × g and 1 ml of the supernatant was added to 1 ml of a medium containing 120 mM Tris, 25 mM Mg^{2+} and 500 μM $NADP^+$ (final pH 7.5). The increase in the absorption at 340 nm by the medium caused by the addition of 0.7 units of glucose 6-phosphate dehydrogenase per 2 ml was used to calculate the amount of ATP formed during the time of NADH oxidation.

Phosphorylation of 8-azido-ADP was measured by incubating 214 μg of phosphorylating MnMgATP submitochondrial particles in the dark in 10 ml of a medium containing 250 mM sucrose, 10 mM succinate, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mg/ml bovine serum albumin, 10 mM potassium phosphate, 1 mM 8-azido-ADP, 7 mM AMP, 3 μM rotenone, 13 U catalase and 10 μl 10% H_2O_2 (final pH 7.5) Incubation temperature was 30°C. After each 5 and 10 min of incubation time, 10 μl of 10% H_2O_2 and 50 μl 2M potassium-succinate (pH 7.5) were added, respectively. After several incubation periods 1 ml sample was taken and quenched in 300 μl of ice-cold 14% perchloric acid. After centrifuging the samples for 2 min at 15000 × *g*, 1 ml of the supernatant was neutralized with 6 M KOH/0.3 M Mops and centrifuged for another 2 min at 15000 × *g*. 1 ml of the supernatant was added to 1 ml of a medium containing 75 mM Tris, 15 mM $MgCl_2$, 7.5 mM EDTA, 375 μM $NADP^+$, 80 mM glucose and 1.4 units glucose 6-phosphate dehydrogenase (final pH 7.5). The difference between the absorption at 340 nm before and 45 min after the addition of 7 units hexokinase (for which 8-azido-ATP acts as a substrate) per 2 ml was used to calculate the amount of triphosphate formed.

SDS-polyacrylamide gel electrophoresis was carried out on long cylindrical gels essentially as described by Swank and Munkres [12] with the modifications that the acrylamide-to-bisacrylamide ratio was raised to 30:1, and that β-mercaptoethanol was omitted from the dissociation buffer. Furthermore, solution B was freshly prepared each time and SDS (0.1%) was added to the upper buffer as a solid shortly before each run. The total polyacrylamide concentration in the gel was 7%. Up to 400 μg of submitochondrial particles protein could be applied to a gel without seriously affecting the separation between the α and the β subunits of the F_1.

After running, the gels were stained for 8 h at 50°C in 0.2% Coomassie brilliant blue, 50% methanol and 7% glacial acetic acid. The gels were destained at 50°C in 20% methanol and 10% glacial acetic acid, and scanned at 560 nm using a Zeiss gel scan spectrophotometer. Then the gels were frozen with solid carbon dioxide and cut into 1 mm slices using a Mickle gel slicer. To these slices 1 ml of a 9:1 Protosol/water mixture was added. After incubation for 24 h at 37°C, 200 μl glacial acetic acid and 4 ml scintillation liquid were added to each vial. After standing in the dark for another 24 h, radioactivity was measured by counting for 10 min in a Packard tricarb liquid scintillation spectrometer. The background radioactivity, usually around 100 dpm, was substracted for each vial.

F_1 content of the submitochondrial particles.
The concentration of F_1 in the submitochondrial particles preparations was determined by measuring the concentration of antimycin-binding sites, since this is equal to that of F_1 in this type of particles [13]. The concentration of antimycin-binding sites was determined by titrating 5–6 mg particles with antimycin in a medium containing 250 mM sucrose, 5 mM EDTA, 1 mg/ml bovine serum albumin and 10 mM Hepes-KOH (pH 7.5) and measuring the fluorescence [14] with an Eppendorf 1101 M fluorimeter. In the submitochondrial particles preparations this value (equal to that of the QH_2 : cytochrome *c* oxidoreductase) was usually about 0.33 nmol/mg protein. If we assume a molecular weight of 368 000 for the F_1, 0.33 nmol F_1/mg submitochondrial particles is equal to 12% of the total submitochondrial protein.

The ATPase activity of inhibitor-free submitochondrial particles, as measured in the presence of uncoupler and 10 mM of the activating anion HCO_3^-, was found to be 16–18 U/mg submitochondrial particles. This corresponds to 142 units of ATPase activity per mg F_1, a value which is found by many authors for isolated F_1 [15,16].

Results

8-Azido-ATP as a substrate for the ATP synthase

As shown in Fig. 1, 8-azido-ATP is hydrolyzed by submitochondrial particles, largely devoid of the F_1 inhibitor, and in the absence of stimulatory anions with the same kinetic pattern as the natural substrate ATP. This also holds for particles still containing endogenous inhibitor. The main difference between the two substrates is the V_{max}, 8-azido-ATP being hydrolyzed at a rate of 300 nmol/min per mg, which is 6% of the rate of ATP hydrolysis in these particles. The K_m values calculated from the Lineweaver-Burk plot are 16 and 230 μM, compared to 30 and 230 μM for ATP hydrolysis. According to Eadie-Hofstee plots the low K_m values are 5 and 10 μM, respectively. The Hill coefficients, derived from a Hill plot (not shown), are 0.73 for 8-azido-ATP and 0.80 for ATP, respectively.

Obviously, in submitochondrial particles in the absence of bicarbonate 8-azido-ATP behaves the same as ATP. We therefore reexamined the hydrolysis of 8-azido-ATP by isolated F_1, since it has not been reported whether the hydrolysis of 8-azido-ATP by isolated F_1 in the absence of bicarbonate is non-cooperative. In the presence of bicarbonate the K_m for 8-azido-ATP is 3-times the K_m for ATP [2]. In Fig. 2 it is shown that in the absence of bicarbonate also in isolated F_1 the hydrolysis of 8-azido-ATP shows cooperative behaviour. This implies that the reported [2] proportionality between saturation with 8-N-ATP and inhibition of ATPase is not due to an absence of cooperativity in the hydrolysis of 8-azido-ATP. The low K_m values for ATP and 8-azido-ATP are

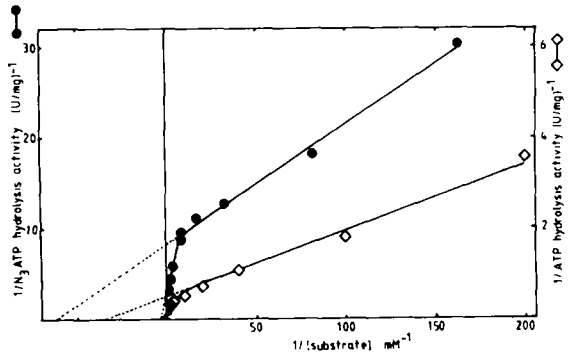

Fig. 1. Kinetics of ATP and 8-azido-ATP hydrolysis by submitochondrial particles in the absence of bicarbonate. ATP was hydrolyzed by 74 μg submitochondrial particles, and 8-azido-ATP was hydrolyzed by 552 μg submitochondrial particles under the experimental conditions described in Materials and Methods for the K_m determinations, except that the pyruvate-kinase concentration was raised to 10 U pyruvate kinase/ml.

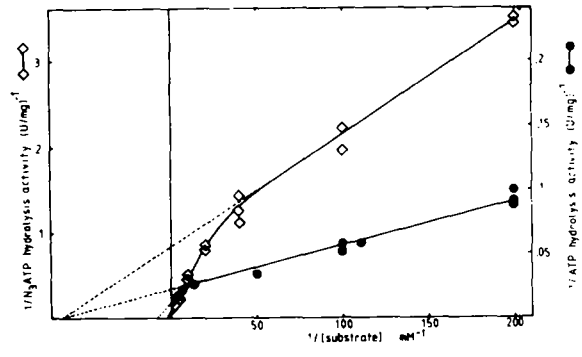

Fig. 2. Kinetics of ATP and 8-azido-ATP hydrolysis by isolated F_1-ATPase (2.4 and 22.5 μg, respectively) in the absence of bicarbonate. The pyruvate-kinase concentration was 10 U pyruvate kinase/ml.

identical, but between the high K_m values a difference of a factor of 3 is found. The Hill coefficients for 8-azido-ATP and ATP (0.87 and 0.73, respectively) are similar to those found with submitochondrial particles. Bicarbonate, known to remove the apparent negative cooperativity of the ATP hydrolysis by isolated F_1 [17], has a similar effect when 8-azido-ATP is used as a substrate for submitochondrial particles (Fig. 3). At the concentration of bicarbonate used hydrolysis of ATP is still cooperative. It should be mentioned that in bovine heart F_1 the K_m found in the presence of bicarbonate equals the high K_m value in the absence of bicarbonate, while in yeast F_1 in the presence of an activating anion (sulphite) the K_m is equal to the low K_m in the absence of such anion [18,19].

8-Azido-ADP as substrate for phosphorylation

Since 8-azido-ADP and 8-azido-ATP do not exchange with tightly bound ADP or ATP, it is important to establish whether 8-azido-ADP can be phosphorylated. This would exclude a catalytic involvement of the tightly bound nucleotides in phosphorylation [20,21]. Although the 8-azido-ADP was highly purified after synthesis, a contamination with 2.0% ADP was measured by reaction with an amount of pyruvate kinase enough to phosphorylate ADP rapidly, but 8-azido-ADP only slowly. Thus, in an experiment where 1 mM 8-azido-ADP was used as substrate for phosphorylation, up to 20 μM ADP might have been present

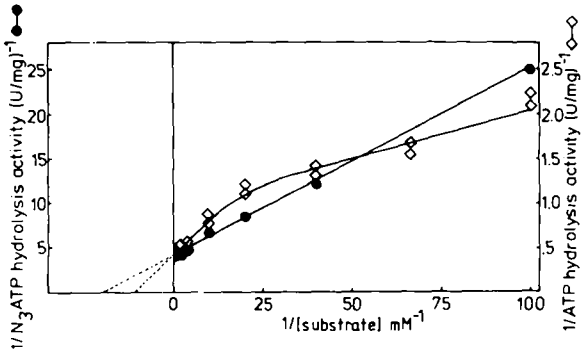

Fig. 3. Kinetics of ATP and 8-azido-ATP hydrolysis by submitochondrial particles, 43 μg and 130 μg, respectively, in the presence of 10 mM bicarbonate. The pyruvate-kinase concentration was 10 U pyruvate kinase/ml.

(for details of the experiment see Methods). In agreement with this a relatively fast formation of about 20 μM triphosphate within the first 10 min of incubation was observed. After this fast phase, a very slow steady-state rate of phosphorylation was reached, leading to about 40 μM triphosphate after 40 min. Oligomycin completely inhibited triphosphate formation, thereby excluding the possibility that enzymes other than the ATPsynthase, e.g., myokinase, were responsible for this phosphorylating activity. It must be concluded, therefore, that 8-azido-ADP is phosphorylated by submitochondrial particles at a very low rate (about 2 nmol 8-azido-ATP/min per mg protein in this experiment). To test the affinity of 8-azido-ADP for its phosphorylating binding site on the membrane-bound F_1, we determined the K_i of 8-azido-ADP for phosphorylation of ADP. This can easily be done, since the rate of phosphorylation of 8-azido-ADP is so low that the triphosphate formed in the presence of both ADP and 8-azido-ADP can be considered to be ATP. From Fig. 4 the K_i is calculated to be about 1 mM, quite different from the K_m of ADP (30 μM). This high value of the K_i of azido-ADP suggests that 8-azido-ADP

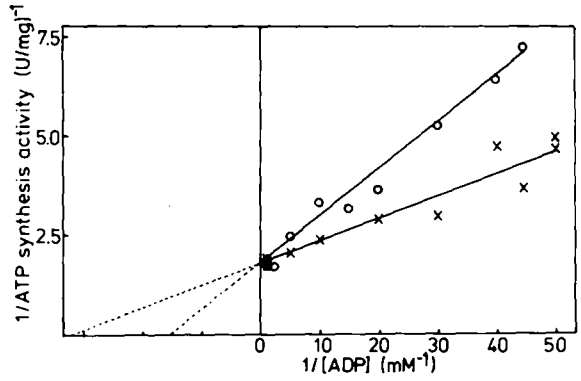

Fig. 4. Effect of 8-azido-ADP on oxidative phosphorylation. 184 μg submitochondrial particles were incubated at 30°C in the dark in an oxygraph vessel. NADH oxidation and ATP synthesis were measured as described in the methods. × ——— ×, no 8-azido-ADP; O ——— O, 1 mM 8-azido-ADP present. The ADP and 8-azido-ADP concentrations were corrected for the 2% contamination of the 8-azido-ADP with ADP. In both experiments the V_{max}, as calculated by linear regression on a Eadie-Hofstee plot, was found to be 550 nmol ATP/min per mg protein. In the presence of 1 mM 8-azido-ADP the K_m for ADP was found to increase from 31 to 67 μM.

cannot bind well to the site involved in phosphorylation. As the K_i of 8-azido-ADP for the hydrolysis of ATP, measured in the presence of 50 mM bicarbonate, was also found to be about 1 mM (not shown), the site of inhibition is probably the same in both reactions.

Labelling of submitochondrial particles with 8-azido-ATP and 8-azido-ADP

The first point to establish is whether inhibition of ATPase activity with covalently bound 8-N-ATP is really due to binding of 8-azido-ATP to the catalytic site for ATP. In Table IA the activity of a sample illuminated for 30 min in the presence of 0.5 mM 8-azido-ATP and 5 mM EDTA is given at various concentrations of ATP. It is clear that ATP protects against the inactivation by 8-azido-ATP, showing that the latter binds to a site to which also ATP can bind. Another competition experiment using 8-azido-ADP, with ADP as competitor, is shown in Table IB. In the absence of ADP maximal inactivation (about 65%) after two incubations is obtained with about 200 μM 8-azido-ADP, which is less than that needed for isolated F_1. When high concentrations of 8-azido-[2-^{3}H]ADP or 8-azido-[2-^{3}H]ATP (1–2 mM) are used, the concentration of bound nucleotides exceeds the concentration of total binding sites present on F_1, even when the inhibition is not complete. Under these conditions not only the ATPase, but also the NADH oxidation and succinate oxidation become inhibited. This aspecific binding is absent when concentrations below 200 μM are used.

Figs. 5A and B show that there is a proportionality between binding of [^{3}H]N-ATP and [^{3}H]N-ADP, respectively, to membrane-bound F_1 and inhibition of ATP hydrolysis, 100% inhibition corresponding to the binding of 2 mol 8-N-ADP or 8-N-ATP per mole F_1. Using lower concentrations of submitochondrial particles during the incubation a higher saturation of F_1 could be reached,

TABLE I

PROTECTION BY ATP AND ADP AGAINST PHOTOINACTIVATION BY 8-AZIDO-ATP AND 8-AZIDO-ADP

(A) Effect of ATP on photoinactivation by 8-azido-ATP of the ATPase activity of submitochondrial particles. ATPase activities were calculated relative to control samples that were co-illuminated in the presence of 500 μM ATP (A) or 500 μM ADP (B). Preincubation of submitochondrial particles with Mg^{2+} and ADP did not cause inhibition of the ATP hydrolysis activity per se. Submitochondrial particles were illuminated for 30 min in the presence of 500 μM 8-azido-ATP, 5 mM EDTA and various concentrations of ATP as described in Materials and Methods. (B) Effect of ADP on photoinactivation by 8-azido-ADP of the ATPase activity of submitochondrial particles. These particles were illuminated with near-ultraviolet light twice for 20 min in the presence of various concentrations of 8-azido-ADP and 5 mM Mg^{2+}. To a parallel incubation an additional 5 mM ADP was added. After illumination the samples were centrifuged through Penefsky columns and ATP-hydrolysis activities were measured as described in Materials and Methods.

Addition	ATPase activity (%)	
A		
None	64	
100 μM ATP	81	
250 μM ATP	84	
500 μM ATP	91	
1000 μM ATP	105	
2000 μM ATP	106	
B	no ADP added	5 mM ADP added
25 μM 8-azido-ADP	69	93
50 μM 8-azido-ADP	48	88
250 μM 8-azido ADP	39	65
500 μM 8-azido-ADP	35	50
1000 μM 8-azido-ADP	37	48

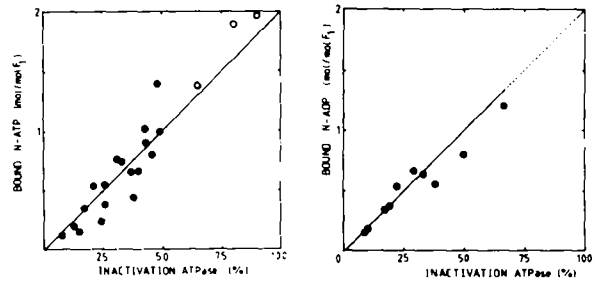

Fig. 5. Photoinactivation of the ATPase activity of submitochondrial particles as a function of the amount of covalently bound 8-N-ATP (A) or 8-N-ADP (B). Determination of bound photolabel and ATPase activity was carried out as described in Materials and Methods. The 8-azido-ATP and 8-azido-ADP concentrations used were in the range 25–100 μM, and 25–125 μM, respectively. The open circles are data from an experiment referred to later.

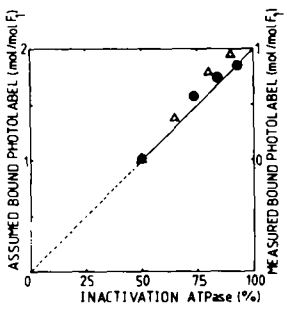

Fig. 6. Photoinactivation of the ATPase activity of submitochondrial particles as a function of additionally bound photolabel to prelabeled submitochondrial particles. Submitochondrial particles were prelabeled with non-radioactive 8-azido-ATP by incubation under near ultraviolet light in the presence of 100 μM 8-azido-ATP and 5 mM EDTA until the ATP-hydrolysis activity was reduced to 50%. The submitochondrial particles were sedimented by centrifugation for 30 min at 150000 × g and the pellet was resuspended in 250 mM Sucrose/10 mM Hepes (pH 7.5). To one half of this preparation 5 mM EDTA and 100 μM 8-azido-[2-^{3}H]ATP (57000 dpm/nmol) were added and to the other half 2 mM Mg^{2+} plus 100 μM 8-azido-[2-^{3}H]ADP (57000 dpm/nmol). The further photolabelling was carried out as described in Materials and Methods and the amount of radioactive photolabel and the total photoinactivation were measured. (●) are the data for the additionally bound N-ADP and (△) are the data for the additionally bound N-ATP.

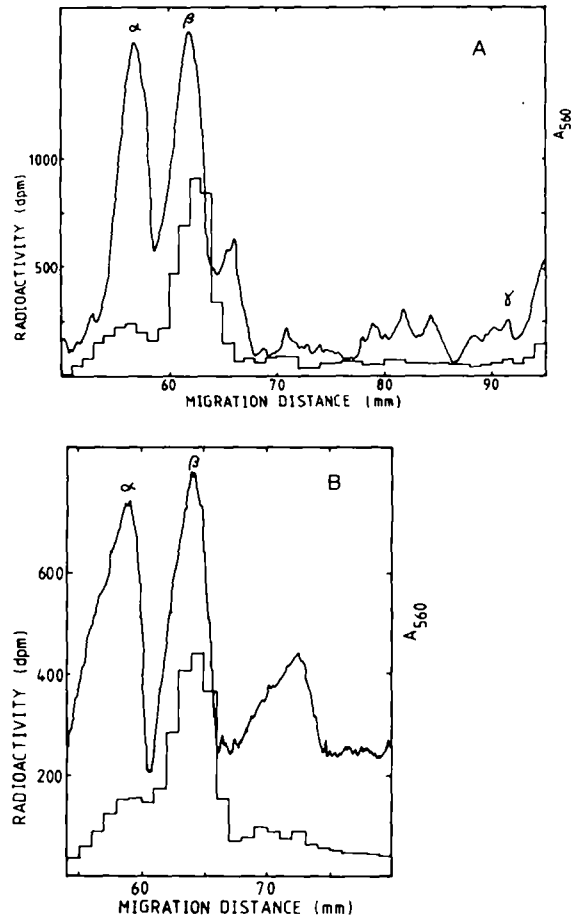

Fig. 7. Distribution of 8-azido-ATP and 8-azido-ADP covalently bound to submitochondrial particles among the protein subunits as separated by SDS-gel-electrophoresis. 210 μg (A) or 300 μg submitochondrial particles (B), photolabelled with 100 μM 8-azido-[2-^{3}H]ATP (58000 dpm/nmol) for three periods of 20 min (A) or with 125 μM 8-azido-[2-^{3}H]ADP (57000 dpm/nmol) for a single period of 20 min (B), were subjected to urea-SDS gelectrophoresis as described in Materials and Methods. The radioactivity in the slices is given by the histogram and the continuous line gives the absorption at 560 nm by the Coomassie brilliant blue stain.

but it did not exceed 2. Also when particles were first incubated with 8-azido-ATP to reach 50% inactivation (1 mol 8-N-ATP/mol F_1 bound) and afterwards with 8-azido-ADP (Fig. 6) no more than two sites were labelled and the proportionality between inhibition and binding remained. This result is in contrast with the data obtained with isolated F_1, in which two sites can be additionally saturated with 8-N-ADP when two sites are already occupied with 8-N-ATP [2]. A second difference between isolated F_1 and that present in submitochondrial particles is the localization of the bound label, which in submitochondrial particles is the same both with 8-azido-ATP in the presence of EDTA and with 8-azido-ADP in the presence of Mg^{2+} (Fig. 7A and 7B). This distribution was independent of the level of saturation. Also in the experiment of Fig. 6 the same distribution of both 8-N-ADP and 8-N-ATP was found. On the average 77% (±2% (S.D.)) was bound to the β subunits and 23% to the α subunits. The specificity is also illustrated in Fig. 7A. The band running just before the β subunit (core protein of the QH_2 : cytochrome c oxidoreductase) and the γ subunit of F_1 are not labelled. The preferential binding of 8-N-ADP to the β subunits was also demonstrated by an autoradiogram (not shown). Both the number of binding sites for 8-N-ADP

and the localization of the bound 8-N-ADP were found to be independent of the presence of Mg^{2+} or EDTA.

To confirm the higher affinity of membrane-bound F_1 for 8-azido-ATP and 8-azido-ADP compared with isolated F_1, as revealed by the concentration needed for maximal labelling, a binding experiment was carried out in which submitochondrial particles were mixed with twice as much isolated F_1 as present on the particles and were irradiated in the presence of 100 μM 8-azido-ADP. As seen in Fig. 8, the F_1 on the particles contained much more nitreno-ADP than the isolated F_1. Additionally, we found in this experiment that the distribution of the label between α and β subunits was different in the particles and in isolated F_1. In the latter preparation the label was nearly exclusively bound to the β subunits.

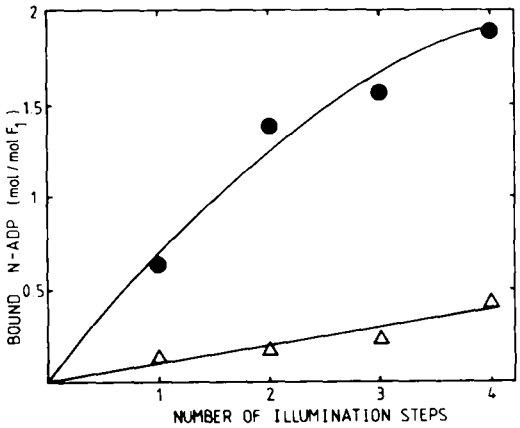

Fig. 8. Binding of 8-azido-ADP to membrane-bound F_1 and to isolated F_1. Submitochondrial particles and F_1 were mixed in the Mg^{2+}-containing illumination buffer at final concentrations of 8 and 2 mg/ml, respectively. To this suspension 100 μM 8-azido-[2-^{3}H]ADP was added, followed by four illumination cycles of 20 min each (at room temperature) as described in Materials and Methods. After each cycle a sample was taken and centrifuged for 35 min at 150000 × g in order to separate the soluble from the particulate F_1. The pellet was resuspended in the Mg^{2+}-containing buffer and showed an ATP-hydrolysis activity with an oligomycin sensitivity of 90–95%. The supernatant was precipitated by adding ammoniumsulphate to 50% saturation and centrifugation for 4 min at 15000 × g. These F_1-pellets were resuspended in the Mg^{2+}-containing buffer. These samples were subjected to urea-SDS gel electrophoresis and the amount of covalently bound radioactive 8-N-ADP in the $\alpha\beta$-region was determined as described in Materials and Methods. (△) are the data for the soluble F_1 and (●) are for the membrane-bound F_1.

Kinetics of ATP hydrolysis by submitochondrial particles

The apparent cooperativity of the catalytic sites of F_1 as revealed by steady-state kinetic measurements can be explained in several ways:
(1) the presence of two independent catalytic sites with different affinities for ATP;
(2) cooperativity between two identical sites;
(3) cooperativity between two sites operative at low ATP concentrations and a third site operative at high ATP concentrations [22]; or
(4) the influence of a regulatory site on the K_m of the catalytic site(s) [18,19]. It has to be kept in mind that the on/off cooperativity between two catalytic sites as shown by Grubmeyer et al. [23] is a different kind of cooperativity: they showed that the activity of each of the two catalytic sites is dependent on whether or not the other site is also occupied by a nucleotide. Under the conditions of catalysis both sites are occupied even at the lowest concentrations of ATP or 8-azido-ATP used. (Enzyme molecules with one site occupied do not show a measurable turnover.) Since the concentration of 8-azido-ADP and 8-azido-ATP used in the inactivation experiments is less than the high K_m value for 8-azido-ATP, the high affinity site would be mainly inactivated if indeed two different sites are present (possibility 1). However, the data of Fig. 9 show that partial inactivation does not change the cooperative characteristics of the Lineweaver-Burk plot. In addition, functional cooperativity between two identical sites (possibility 2) can be excluded on the basis of the proportionality between saturation of the two sites with inhibitor and the inhibition of ATPase activity. The presence of a third site, only saturated at high concentrations under which conditions all three sites obtain a high K_m (possibility 3), is also excluded by these data, unless it is assumed that upon inhibition of two sites the third site is no longer operative. The proportionality between saturation with ligand and inhibition of ATPase activity excludes any mechanism in which the activity of each of the two sites that can be labelled is dependent on the catalytic functioning of the other (possibility 2 and 3). The possibility that each F_1 molecule contains either two analogue molecules or none can be excluded, since even if the binding were highly cooperative, the covalent attachment

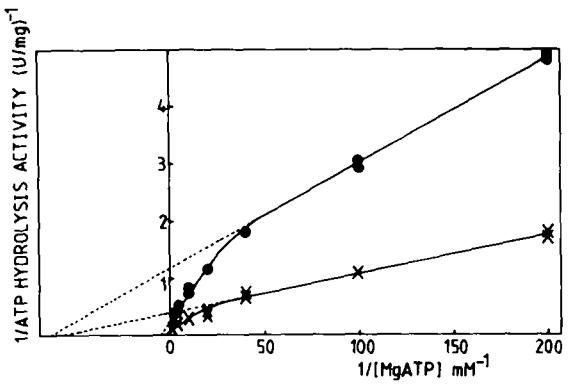

Fig. 9. Kinetics of ATP hydrolysis by control and partly inhibited submitochondrial particles. A batch of inhibitor depleted submitochondrial particles was incubated under near ultraviolet light in the presence of 100 μM 8-azido-ATP until the ATP-hydrolysis rate was inhibited by 50%. After removal of the (un)photolysed label on a Penefsky column, equilibrated with the Mg^{2+}-containing illumination buffer, K_m was determined as described in Materials and Methods. Exactly the same procedure was carried out with a control sample, except that the 100 μM 8-azido-ATP was replaced by 100 μM ATP. The data are plotted in a Lineweaver and Burk plot, (×) being the data for the control sample and (●) being the data for the partly inhibited submitochondrial particles.

is not (see also Discussion). It has to be concluded therefore, that only regulation by another site, not able to bind 8-azido-ADP or 8-azido-ATP in the absence of turnover (possibility 4), can explain the cooperative kinetics. In Fig. 10 two simulations are shown, one based on the Recktenwald and Hess model [18] in which the affinity of the catalytic sites for the substrate is regulated by the saturation of a regulatory site with the substrate, while the V_{max} remains unchanged. When K_{m_1} is the K_m for ATP of those F_1 molecules of which the regulatory binding site is not occupied with ATP and K_{m_2} the K_m when this site does contain ATP, the overall ATP-hydrolysis reaction rate V is given by the equation:

$$V = \frac{V_m[ATP]}{K_{m_1}+[ATP]} \frac{K_d}{[ATP]+K_d} + \frac{V_m[ATP]}{K_{m_2}+[ATP]} \frac{[ATP]}{[ATP]+K_d},$$

K_d being the dissociation constant of ATP bound to the regulatory binding site.

The other simulation is based on the model for two different catalytic sites, in which the total reaction rate V equals the sum of the two separate reaction rates, according to the equation:

$$V = \frac{V_{max_1}[ATP]}{K_{m_1}+[ATP]} + \frac{V_{max_2}[ATP]}{K_{m_2}+[ATP]},$$

K_{m_1} and V_{max_1} being the K_m and V_{max} for the high affinity reaction and K_{m_2} and V_{max_2} being the K_m and V_{max} for the low affinity reaction respectively. In fig. 10 this model is referred to as the "double Michaelis-Menten model". Both models give a reasonable fit. In the K_m regulation model, a value of 32 μM for the K_d of the complex between the regulatory site(s) and ATP gives the best fit.

When the incubation in the light is carried out in the presence of 250 μM 8-azido-ATP which should be high enough to saturate any low-affinity site and the regulatory site, but the time of illumination is such that the V_{max} of ATP hydrolysis is inhibited 50%, just as in the experiment of Figure 9, the Lineweaver-Burk plot (not shown) is

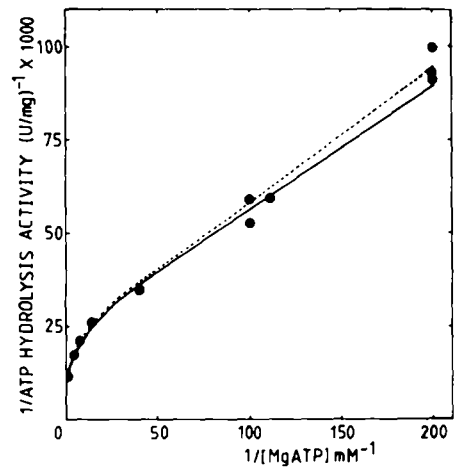

Fig. 10. Simulated kinetics of ATP hydrolysis by isolated F_1-ATPase. ATP hydrolysis was carried out as described in Methods and Materials and the data were plotted in a Lineweaver-Burk plot. (●) are the experimental data, the discontinuous line being calculated according to the 'K_m-regulation model' with K_{m_1} = 35 μM, K_{m_2} = 200 μM, K_d(R-MgATP) = 32 μM and V_{max} = 100 U/mg as fitting parameters and the continuous line being calculated according to the 'double Michaelis-Menten model' with K_{m_1} = 12 μM, K_{m_2} = 350 μM, V_{max_1} = 35 U/mg and V_{max_2} = 65 U/mg as fitting parameters.

still identical to that of Fig. 9. This indicates that, when there is no turnover, the regulatory binding sites cannot be photolabelled.

Discussion

The data reported in this paper confirm previous results with isolated F_1 indicating that 8-azido-ATP is a useful analogue for ATP as substrate for hydrolysis. From our experiments it can be deduced that also in submitochondrial particles the binding sites of 8-azido-ATP are the same as the catalytic sites of F_1. 8-Azido-ADP binds to these same sites under the conditions of the labelling experiments (no turnover). The findings that 8-azido-ADP in the presence of Mg^{2+} does not bind to the α subunits of F_1 as has been reported for isolated F_1 indicates that the structure of isolated F_1 differs from that of F_1 in the particles. We do not know, however, whether 8-azido-ADP binds to the α subunits in submitochondrial particles under conditions different from those used in the experiments reported in this paper, e.g., at high concentrations of 8-azido-ADP. Such experiments could not be carried out successfully because of the aspecific binding of 8-azido-ADP to various polypeptides in the particles, among which are the F_1 subunits.

It may be significant that under our conditions of labelling (100 μM 8-azido-2[^{3}H]ADP) the 8-N-ADP, bound to isolated F_1, is nearly exclusively located on the β subunits in contrast to the results of Wagenvoord et al. [1,2] obtained at higher label concentrations. More detailed studies on the binding of 8-azido-ADP to isolated F_1 will be reported elsewhere.

It is noteworthy that the difference between the localisation of the photolabel in membrane-bound and isolated F_1, obtained in the experiment of Fig. 8, is also found by Shavit and coworkers [24,25] who used 3′-*o*-(4-benzoyl)benzoyl ADP to label CF_1. However, since the differences between CF_1 and F_1 in respect to the catalytic and regulatory binding sites are not yet clear, it is not possible to assign a certain pattern of labelling to a certain type of site. The observed differences in binding characteristics between isolated and membrane-bound F_1 could be due to differences in affinity of the various sites as well as to differences in conformation of isolated and membrane-bound F_1.

The distribution of bound 8-N-ADP and 8-N-ATP over the α and β subunits can be explained by the assumption [26–28] that the binding site is on the β subunit, at the interface between α and β, so that on photo-inactivation some of the nitreno radical formed finds an amino acid of the α subunit to react with, instead of an amino acid of the β subunit to which the nucleotide is largely attached.

The clearly linear relationship between saturation of the F_1 and inhibition of ATP hydrolysis (also of ATP synthesis [29]) indicates that, with respect to nucleotide binding, both for hydrolysis and synthesis of ATP the sites on the β subunit work independently and non-cooperatively. Only cooperativity between catalytic and regulatory sites will occur. This non-cooperativity does not mean that the properties of each of the two (catalytic) sites are independent of the fact whether the other site is occupied with a nucleotide or not, but it means that when one site is occupied the other is catalytically active. The conclusions of Grubmeyer et al. [23] are interpreted by us in such a way that not only reversibly bound ligands at one site modify the properties of the second site, but also irreversibly bound ligands. Each site is fully competent for catalysis, provided the other site contains a bound nucleotide. Turnover at this latter site is not requirued. The presence of an interaction between regulatory and catalytic sites is evident from the shape of the Lineweaver-Burk plots for ATP hydrolysis and from the fact that 8-N-ADP bound to the regulatory sites (in isolated F_1) inhibits the binding of 8-azido-ATP to the catalytic sites and hydrolysis of ATP [1,2]. We then favor the assumption that binding of ATP to the regulatory binding site(s) during hydrolysis of ATP increases the K_m of ATP for its binding site on the catalytic subunit. We do not exclude the possibility that during hydrolysis or synthesis of ATP the α and β subunits are both catalytically involved, as is proposed by Kozlov and Skulachev [31].

What does not fit with our interpretation of the data is the idea that the mechanism is sequential [22,30]. In a sequential mechanism the irreversible occupation of one site with 8-N-AXP blocks all catalytic activity. A sequential mechanism would still be possible if in our experiments each F_1

molecule contains either none or two molecules bound 8-N-AXP. This possibility can easily be excluded. During our labeling experiments, with label concentrations much higher than the K_d of the catalytic sites for 8-azido-AXP, both catalytic sites are fully occupied. This abolishes any effect of a possible binding cooperativity on the distribution of (non-covalently) bound 8-azido-AXP among the F_1-molecules. Furthermore, the covalent attachment of any bound 8-azido-AXP to the protein is determined by statistics. At an average of 1 mol 8-N-AXP bound per mol F_1 the following statistical distribution of the ATPase molecules will occur: 25% of the F_1 molecules will have no label bound, 50% will have one 8-N-AXP bound and 25% will have both catalytic binding sites occupied. The covalent attachment by irradiation will show no discrimination in favour of nucleotides bound to an F_1 molecule that contains already a covalently attached nucleotide. Thus the simplest mechanism appears to be catalysis at one of the two catalytic sites on the β-subunits (while the other contains a nucleotide as well), an additional site regulating the affinity of this catalytic site for its substrate. When ATP is bound to the regulatory site the K_m of the catalytic site is increased to 230 μM. According to the model of Recktenwald and Hess, proposed on basis of the data with yeast F_1 [18,19], other anions like SO_4^{2-}, HSO_3^- and HCO_3^- bind to the same regulatory site.

An alternative interpretation of the data is that when we bind two moles of nitreno-AXP per mol F_1 it could be that we are dealing with two sites, both occupied to the same extent with 8-azido-AXP during the illumination, but one being catalytic for ATP hydrolysis and the other not. Although different affinities for the photolabels would be expected if this were the case, the two sites could not be discriminated in submitochondrial particles within the concentration range that could be studied (25–125 μM 8-azido-AXP). If only one of the two sites labelled with N-AXP is involved in ATP hydrolysis a sequential mechanism is consistent with the data. In that case it has to be assumed not only that the second site has nearly the same affinity for 8-azido-ADP and 8-azido ATP as the one involved in ATP hydrolysis, but also that occupation of the second site has no effect on the V_{max} of the ATP-hydrolysis activity. Since more than one site is involved in the catalytic process it would mean that only one of the sites in this catalysis can be labelled with up to 125 μM 8-azido-ATP or 8-azido-ADP.

8-Azido-ATP is found to be a good substrate for particle-bound F_1 with the same affinity for the catalytic site as ATP, and also 8-azido-ADP binds to this site with a similar affinity. However, 8-azido-ADP is a very inefficient inhibitor of both ATP synthesis and ATP hydrolysis ($K_i \approx 1$ mM). This result not only indicates that synthesis and hydrolysis of ATP occur at the same site, but also that during turnover (in any direction) the conformation of this catalytic site changes so much that only nucleotide triphosphates and a certain type of nucleotide diphosphates (type I nucleotides [20]) are bound efficiently. The data of Roveri et al. [15] show that during the first seconds of hydrolysis of ATP, the affinity for ADP increases, also indicative of the proposed conformational change. The fact that inhibition of ATP hydrolysis by ADP remains competitive after this change of K_i shows that the site of inhibition for ADP is in fact the catalytic site and not a special regulatory site. The very slow rate of phosphorylation of 8-azido-ADP can be very well related to the very low affinity for 8-azido-ADP of the catalytic site under turnover conditions. In further experiments we will investigate whether 8-azido-ATP can bind to additional (regulatory) sites under conditions of turnover.

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