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LOCALISATION OF ADENINE NUCLEOTIDE-BINDING SITES ON BEEF-HEART MITOCHONDRIAL ATPase BY PHOTOLABELLING WITH 8-AZIDO-ADP AND 8-AZIDO-ATP

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Summary

1. In addition to the previously studied 8-azido-ATP, 8-azido-ADP is a suitable photoaffinity label for beef-heart mitochondrial ATPase (F_1).

2. Photolysis at 350 nm of 8-azido-ADP in the presence of isolated F_1 leads to inactivation of ATPase activity. Both ATP and ADP (but not AMP) protect against the inactivation.

3. In the absence of Mg^{2+}, 8-azido-ADP binds almost equally to the α and β subunits of F_1, whereas in the presence of Mg^{2+} the α subunits are predominantly labelled.

4. The ATPase activity is completely inhibited when two molecules of 8-azido-ADP are bound per molecule F_1.

5. 8-Azido-ATP and ATP are competitive substrates for F_1, indicating that in the presence of Mg^{2+} 8-azido-ATP binds to the same site as ATP.

6. The amount of tightly bound nucleotides in F_1 is not significantly changed upon incubation with 8-azido-ATP either in the light or the dark.

7. 8-Azido-ATP is also a suitable photoaffinity label for F_1 in the isolated ATPase complex and submitochondrial particles, photolabelling leading to inactivation of ATPase activity.

8. As is the case for isolated F_1 the α and/or β subunits of F_1 in the ATPase complex or particles are specifically labelled with 8-azido-ATP.

9. Oxidative phosphorylation and the ATP-driven reduction of NAD^+ by succinate are also inhibited by photolabelling Mg-ATP particles with 8-azido-ATP.

10. In contrast to the uncoupled ATPase activity, where the two ATP-binding sites do not interact, cooperation between the two sites is required for ATP hydrolysis coupled to reduction of NAD^+ by succinate.

Introduction

An approach to understanding the mechanism of ATP hydrolysis and ADP phosphorylation in oxidative phosphorylation is to locate the binding sites of substrates and products on the ATPase coupling factor F_1, which catalyses these reactions [1,2]. In an earlier paper [3] we reported the effect of labelling isolated F_1 with the photoaffinity label 8-azido-ATP. In this paper an extension of these experiments, using 8-azido-ADP as well as 8-azido-ATP and membrane-bound as well as isolated F_1, is described.

The ATPase F_1 contains five types of subunit [4,5] designated α, β, γ, δ, and ϵ according to their mobility during polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. Although the function of these subunits is not completely elucidated, there are several indications that the β subunit plays a role in ATPase activity. Aurovertin which inhibits both ATP synthesis [6] and ATP hydrolysis [7,8] binds specifically to the β subunits of F_1 [9]. Russell et al. [10] and Lunardi et al. [11] showed that mitochondrial F_1 is inactivated by irradiation in the presence of arylazido aminopropionyl-ATP and azidonitrophenylaminobutyryl-ADP, respectively. The latter authors found that both the α and β subunits are labelled [11]. Irradiation in the presence of 8-azido-ATP results in the predominant labelling of the β subunit in F_1 from beef-heart [3] and *Micrococcus luteus* [12] and of the α subunit in *Escherichia coli* F_1 [13]. Labelling of the β subunit of mitochondrial F_1 with an alkylating ATP analogue was shown by Budker et al. [14]. Moreover, Esch and Allison [15] have shown strong evidence that labelling of tyrosine residues on the β subunits, in total three, of mitochondrial F_1 with *p*-fluorosulfonyl-benzoyl-5'-adenosine leads to complete inactivation of the ATPase activity. Hulla et al. [16] found that up to 6 mol of 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5'-triphosphate are bound to F_1 from *Micrococcus*, of which 2 mol are covalently bound, both to the β subunits. Reconstitution experiments with isolated subunits from E. coli F_1 [17] and from F_1 from thermophilic bacterium PS3 [18] also strongly indicate that the β subunits are necessary for ATPase activity.

In the intact enzyme, the role of the γ, δ and ϵ subunits is probably to function as binding protein between F_1 and the F_0 part of the ATPase complex and to function as a gate to the proton channel [18].

Evidence for a role of both the α and β subunits in binding ATP and ADP is given in this paper. The relative labelling of the two subunits depends on the presence or absence of Mg^{2+}. Also membrane-bound F_1 has been studied to determine the effect of photolabelling on oxidative phosphorylation.

Another important feature of F_1 is that is contains tightly bound nucleotides [19,20]. The role of these nucleotides is not yet fully understood. In this paper it is shown that the number of tightly bound nucleotides on isolated F_1 is only slightly changed by incubation and photolabelling with 8-azido-ATP.

Methods and Materials

8-Azido-ATP and 8-azido-ADP were prepared by chemical coupling of PP_i or P_i, respectively, to 8-azido-AMP [21]. The yields were 40 and 50%, respec-

tively. 8-Azido-AMP and 8-azido-[2-^{3}H]ATP were prepared from AMP as described earlier [3,22]. 8-Azido-[β-^{32}P]ADP was prepared by chemical coupling of $^{32}P_i$ to 8-azido-AMP. The products were purified on a DEAE-cellulose column by elution with 20—400 mM triethylammonium bicarbonate in a linear gradient. Appropriate fractions were freeze-dried and precipitated as lithium salts in ethanol/acetone (1 : 1, by vol.).

Beef heart mitochondrial ATPase was prepared according to Knowles and Penefsky [23].

Submitochondrial particles (Mg-ATP particles) were prepared from heavy beef-heart mitochondria [24] according to Löw and Vallin [25] and depleted of the ATPase inhibitor according to Van der Stadt et al. [26]. The ATPase complex was prepared from beef heart mitochondria and the activity was measured as described by Berden and Voorn-Brouwer [27].

The ATPase activity (defined as μmol ATP hydrolysed per min per mg protein at 30°C) was measured either spectrophotometrically (by measuring ADP-induced oxidation of NADH in a coupled pyruvate kinase-lactate dehydrogenase system) or by determining liberated P_i according to Fieske and Subbarow as described by Sumner [28], using the reaction medium described previously [3]. The reaction was started with about 1 μg F_1 or 50 μg submitochondrial particles.

The phosphorylation activity was determined at 22°C by measuring esterified $^{32}P_i$ (as glucose 6-phosphate) in an ATP-trapping system, with dialysed hexokinase (25 units/ml) and 50 mM glucose. The reaction medium (pH 7.5) also contained 5 mM KH_2PO_4, 5 mM ADP, 100 mM sucrose, 1 mM EDTA, 75 mM Tris-HCl buffer, 10 mM succinate, 25 mM KCl and about 0.5 μg particles per ml. The reaction was started by adding carrier-free $^{32}P_i$ and 25 mM $MgCl_2$. The reaction was terminated by adding 100 μl reaction mixture to 800 μl 2 M $HClO_4$. Non-esterified $^{32}P_i$ was extracted at 0°C according to Nielsen and Lehninger [29]. $^{32}P_i$ was detected as Cerenkov radiation in a liquid-scintillation counter (ISOCAP).

ATP-driven reduction of NAD^+ by succinate was measured at 30°C according to Ernster and Lee [30].

Tightly bound nucleotides on isolated F_1 were extracted as described by Harris et al. [19]. ATP, ADP, and AMP were measured according to Bergmeyer [31]; ATP and ADP were also determined with the luciferase/luciferin system as described by Stanley and Williams [32]. The emitted light was detected with a CHEM GLOW Aminco Photometer, to which was coupled an Aminco Integrator Timer with printer. ADP was converted to ATP, before measuring.

Soluble protein was measured according to Lowry et al. [33], with bovine serum albumin ($A^{1\%}_{279\,nm} = 6.67$) as standard. Membrane-bound protein was determined according to Cléland and Slater [34].

Photolabelling was carried out as described earlier [3]. ATPase complex and submitochondrial particles were cooled by placing the dish in an ice-water mixture.

Electrophoresis on 12 or 13.5% polyacrylamide gels in the presence of dodecylsulphate was carried out according to Weber et al. [35], using stacking gels as described by Maurer [36]. Further details were described in our previous paper [3]. With 8-azido-[β-^{32}P]ADP-labelled F_1, staining and destaining of the

gels was carried out at 0°C to prevent hydrolysis of the esterified $^{32}P_i$. Gels were scanned at 500—600 nm using a Zeiss (Z.K. 4) gel scanner. The procedure to slice the gels, to extract the gel slices and the determination of radioactivity as ^3H was as described earlier [3]. ^{32}P was determined by dissolving the gel slices in 30% H_2O_2 (by vol.), adding water to the clear solution and detecting the Cerenkov radiation.

DEAE-cellulose was obtained from Serva (Servacel, type DEAE SH), [2-^{3}H]-AMP from the Radiochemical Centre, Amersham, U.K., Protosol from New England Nuclear and $^{32}P_i$ from Philips-Duphar, Petten.

Results

Photolabelling of isolated F_1 with 8-azido-ADP

Fig. 1 shows that on irradiation with ultraviolet light (350 nm) 8-azido-ADP inhibits the ATPase activity of F_1. Addition of ADP protects against the inactivation. Other experiments showed that protection is also given by ATP (slightly less effective than ADP), but not by AMP. Added Mg^{2+} has little effect on the photoinactivation by 8-azido-ADP, but the protection by ADP is somewhat greater in the presence of Mg^{2+}. Irradiation of F_1 in the absence of photolabel did not lead to inactivation of ATPase activity (cf. Ref. 3).

To determine the number and location of the binding sites for ADP, F_1 was photolabelled with 8-azido-[β-^{32}P]ADP in the absence and presence of Mg^{2+} and subjected to electrophoresis on polyacrylamide (13.5%) dodecyl sulphate gels. Fig. 2 illustrates that the α and β subunits are well separated when a small amount (9.1 μg) of F_1 is brought on to the gel. The protein bands were cut out and their radioactivity was determined (cf. Ref. 3). Only the α and β bands showed significant radioactivity above the background.

Table I gives the amount of label bound to each subunit, the total bound to the α plus β subunits and the percentage inactivation of the ATPase activity brought about by the binding. An important observation is that on labelling without added Mg^{2+} both large subunits are labelled (Fig. 2A), whereas when Mg^{2+} is present during photolabelling the α subunit is predominantly labelled

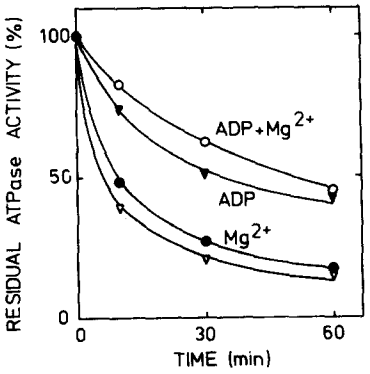

Fig. 1. Photo-inactivation of isolated F_1 by irradiation with ultraviolet light (350 nm) in the presence of 1.7 mM 8-azido-ADP (▽———▽), and effect of 2 mM ADP (▼———▼) and of Mg^{2+} (added in 2 mM excess to the nucleotides) on the inactivation, without (●———●) and with (○———○) ADP present.

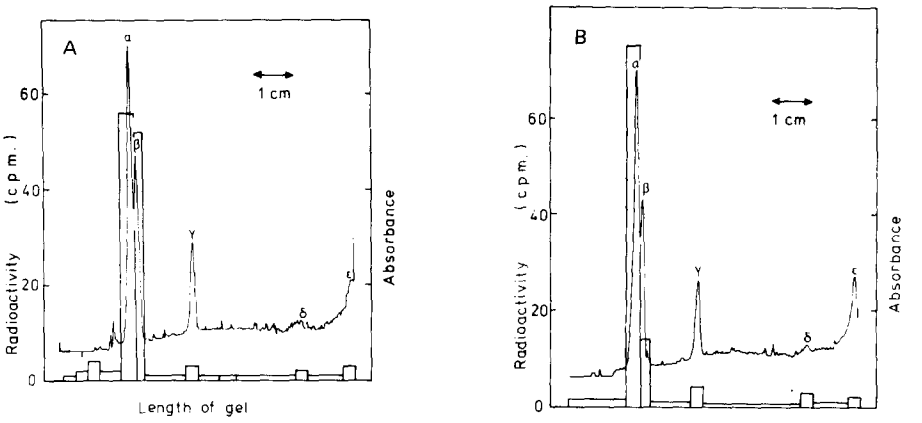

Fig. 2. A scan of a polyacrylamide-dodecyl sulphate gel of 9.1 μg F_1 labelled by 8-azido-[β-^{32}P]ADP in the absence (A) and presence (B) of 2 mM Mg^{2+} in excess to nucleotides. Each histogram gives the radioactivity and the continuous line the absorbance at 540 nm.

(Fig. 2B). Furthermore, it can be concluded that on extrapolation to 100% inactivation 2 molecules of label are bound per molecule under these conditions. Thus at least two binding sites for 8-azido-ADP are present in isolated F_1 under these conditions. In the presence of Mg^{2+} the nucleotide binds to the α subunits, but in the absence of Mg^{2+} it binds to both the α and β subunits.

Competition between 8-azido-ATP and ATP for catalytic site

In our previous study [3] 8-azido-ATP was used only in the absence of Mg^{2+}, since it is hydrolysed slowly by F_1 in the presence of Mg^{2+}. We showed too that ATP protects against inactivation by 8-azido-ATP, indicating that they compete for the same site, but the effect of Mg^{2+} on the labelling by 8-azido-

TABLE I

PHOTOLABELLING BY 8-AZIDO-[β-^{32}P]ADP AND 8-AZIDO-[2-^{3}H]ATP OF THE SUBUNITS OF BEEF-HEART F_1

F_1 was labelled by irradiation for 1 h with 1.7 mM 8-azido-[β-^{32}P]ADP (3270—2940 cpm/nmol) as described in Fig. 1. The adenine nucleotides were added in a concentration of 2 mM. Mg^{2+} was added in an excess of 2 mM to the nucleotides. The amount of bound label was determined by cutting out protein bands in polyacrylamide-dodecyl sulphate gels and measuring the ^{32}P radioactivity. The total amount of radioactivity in the α and β subunits was measured with 54.7 μg F_1 applied to the gel. The label in the α and β subunits separately was determined by applying 9.1 μg protein to the gels (see Fig. 2). The data for bound 8-azido-[2-^{3}H]ATP were taken from the earlier publication [3].

Label and addition	ATPase inactivation (%)	Bound 8-azido-ADP(ATP) (mol/mol F_1) on					
		α	β	αβ	γ	δ	ε
N_3-ADP, —	86	0.76	1.08	1.85	0.04	0.02	0.03
, ATP	57	0.71	1.09	1.47	0.03	0.02	0.01
, ADP	58	0.41	1.12	1.54	0.03	0.01	0.02
, AMP	82	0.76	1.12	1.86	0.03	0.02	0.01
, Mg^{2+}	83	1.53	0.30	1.89	0.06	0.05	0.03
, ADP + Mg^{2+}	53	1.38	0.13	1.42	0.07	0.04	0.03
N_3-ATP, —	88	0.17	1.51	1.68	—	—	—

ADP made it desirable to test if the binding sites for ATP and 8-azido-ATP are identical under conditions of active hydrolysis.

If we assume that F_1 can hydrolyse two nucleotide triphosphates (NTP and NTP*) simultaneously on the same site according to Michaelis-Menten kinetics, we can derive the following formula for total P_i liberation,

$$V_t = \frac{V}{\frac{K_m}{s} + 1}\left[1 - 1/(K_m^*/s^* + K_m^* \cdot s/K_m \cdot s^* + 1)\right] + \frac{V^*}{\frac{K_m^*}{s^*} + 1}\left[1 - 1/(K_m/s + K_m \cdot s^*/K_m^* \cdot s + 1)\right] \quad (1)$$

in which V and V^* stand for the maximal rate of P_i liberation at infinite NTP and NTP* concentrations, respectively, in the absence of the other nucleotide, K_m and K_m^* are the Michaelis constants, s and s^* the concentrations of NTP and NTP*, respectively. Under the conditions used for measuring ATP hydrolysis (with 50 mM HCO_3^- as activating anion [37]), the values were found to be: V, 160 and 14.7 μmol · min^{-1} · mg^{-1}, respectively; K_m, 118 and 500 μM, respectively for ATP and 8-azido-ATP.

Fig. 3 gives reciprocal velocity-concentration plots of the simultaneous hydrolysis of ATP and 8-azido-ATP at a number of fixed 8-azido-ATP concentrations. Theoretical lines calculated to Eqn. 1, in which the above values for V

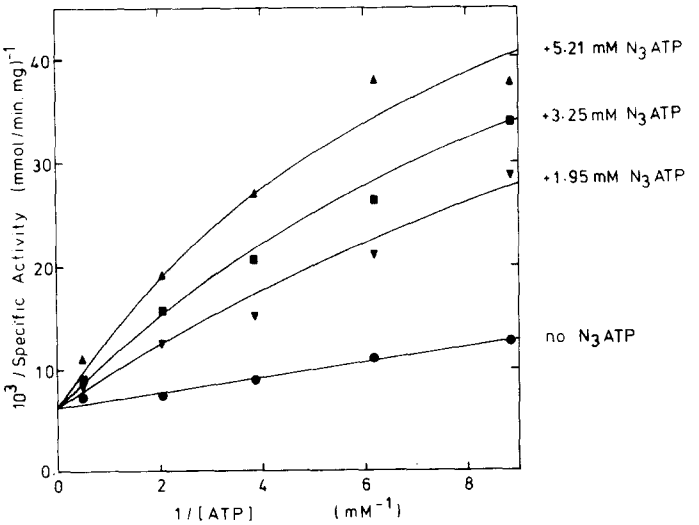

Fig. 3. Double-reciprocal plot of the hydrolysis of ATP by isolated F_1 (●——●). Effect of 1.95 mM (▲——▲), 3.25 mM (■——■) and 5.21 mM (▼——▼) 8-azido-ATP. The drawn lines give the total rate of P_i liberation, assuming that both ATP and 8-azido-ATP are hydrolysed according to Michaelis-Menten kinetics and compete for the same site, and the values for V and K_m given in the text. The reaction was started by adding 0.58 μg F_1 to 300 μl of a solution containing 125 mM sucrose, 15 mM Tris-acetate buffer, 5 mM phospho*enol*pyruvate, 50 mM $KHCO_3$, 1 mM $MgCl_2$, 4.5 units (μmol/min) pyruvate kinase and different concentrations of Mg-ATP and Mg-8-azido-ATP as substrates. The final pH was 8.0 and the reaction temperature 30° C. 90-μl samples were taken at zero 0, 5 and 10 min, and the P_i liberated measured [32].

and K_m are filled in, are drawn. The agreement between the experimental points and the theoretically derived lines justifies the conclusion that ATP and 8-azido-ATP are competitive substrates binding to the same site on F_1 in the presence of Mg^{2+}.

Effect of azido-ATP on amounts of firmly bound ADP and ATP

Table II shows that on incubation and photolabelling of F_1 with 8-azido-ATP the amount of tightly bound nucleotides is scarcely changed. The possibility that tightly bound 8-azido-ATP or 8-azido-ADP could replace the firmly bound nucleotides can be excluded, because the same amount of nucleotides was found with both assay systems [31,32] and it is known that 8-azido-ATP is a substrate for hexokinase, but not for luciferase. These results indicate that the sites of the tightly bound nucleotides differ from those located on the β subunit that can be labelled with 8-azido-ATP [3].

Photolabelling of the ATPase complex and submitochondrial particles with 8-azido-ATP

The ATPase activity of the ATPase complex (containing F_0 as well as F_1) is also inhibited by irradiation in the presence of 8-azido-ATP. ATP but not AMP protects just as was found with isolated F_1. Fig. 4 shows that the $\alpha\beta$ region was labelled with 8-azido-[2-^{3}H]ATP but it is not certain which band is labelled, because the separation of the α and β bands was insufficient.

The ATPase activity, ATP-driven reduction of NAD^+ by succinate and oxidative phosphorylation of submitochondrial particles (pretreated to remove the inhibitory subunit [26]) are all inhibited by photolabelling with 8-azido-ATP. The degree of inhibition of the two coupled reactions is greater than that of the ATPase activity (Table III). In Fig. 5, the degree of inactivation of the ATP-driven reduction of NAD^+ by succinate found after various photolabelling experiments is plotted against the degree of inactivation of the ATPase activity.

TABLE II

EFFECT OF INCUBATION WITH 8-AZIDO-ATP ON AMOUNT OF TIGHTLY BOUND NUCLEOTIDES IN ISOLATED F_1

Four different preparations of F_1 were incubated in the dark (b) or the light (c) with 8-azido-ATP (2 mM) and the content of tightly bound nucleotides measured. The ATPase activity of the F_1 after these treatments is also given.

Amount of nucleotide (mol/mol F_1)						ATPase activity (μmol/min per mg) of		
Not treated (a)		Incubation for 1 h with 8-N_3-ATP						
ATP	ADP	in dark (b)		in light (c)		(a)	(b)	(c)
		ATP	ADP	ATP	ADP			
1.38	0.86	1.34	0.87	1.34	1.05	72.8	64.6	35.1
1.50	1.02	1.15	0.75	1.29	0.75	84.2	78.4	28.7
1.34	1.21	0.89	0.85	0.89	0.75	75.9	79.9	55.1
1.98	0.89	1.40	0.97	1.38	0.96	78.0	75.3	31.7

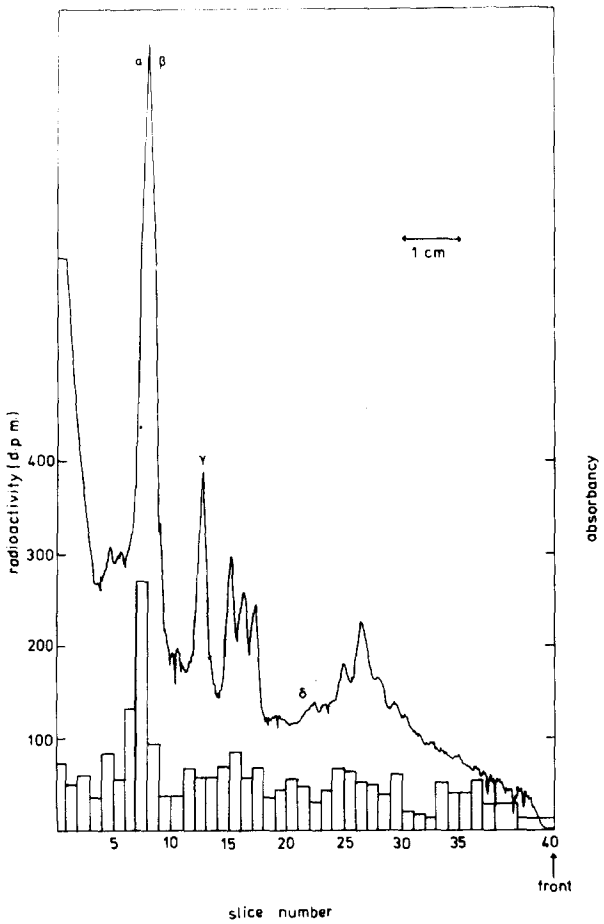

Fig. 4. Polyacrylamide-dodecyl sulphate gel electrophoresis of ATPase complex labelled with 8-azido-[2-^{3}H]ATP. 80 μg were labelled by irradiation for 2 h with 2 mM 8-azido-[2-^{3}H]ATP (10 000 dpm/nmol). It was freed from non-bound label by precipitating the complex 4 times with 50% saturated $(NH_4)_2SO_4$ (pH 7.5) and redissolving in 250 mM sucrose, 10 mM Tris-sulphate buffer (pH 7.5) and 1 mM dithiothreitol. After Coomassie-blue staining the gel was scanned at 500 nm and sliced. The radioactivity of the slices is given by the histogram.

TABLE III

INACTIVATION OF ATPase ACTIVITY, ATP-DRIVEN SUCCINATE-NAD$^+$ REDUCTASE AND PHOSPHORYLATION ACTIVITY OF SUBMITOCHONDRIAL PARTICLES BY PHOTOLABELLING WITH 8-AZIDO-ATP

The particles (inhibitor depleted) were photolabelled by irradiation for 1 h on a dish (diameter 5 cm) at 0°C in the presence of 2 mM 8-azido-ATP.

Treatment	ATPase activity (μmol/min per mg)		Succinate-NAD$^+$ reductase (nmol/min per mg)		Phosphorylation activity (nmol/min per mg)	
	a	b	a	b	a	b
+N_3-ATP dark	3.06	1.99	15.7	—	17.1	13.6
+N_3-ATP light	1.06	0.63	2.0	—	2.4	1.7
+ATP light	2.77	2.20	14.5	—	25.8	21.0
not treated	3.34	2.29	15.6	—	28.3	—

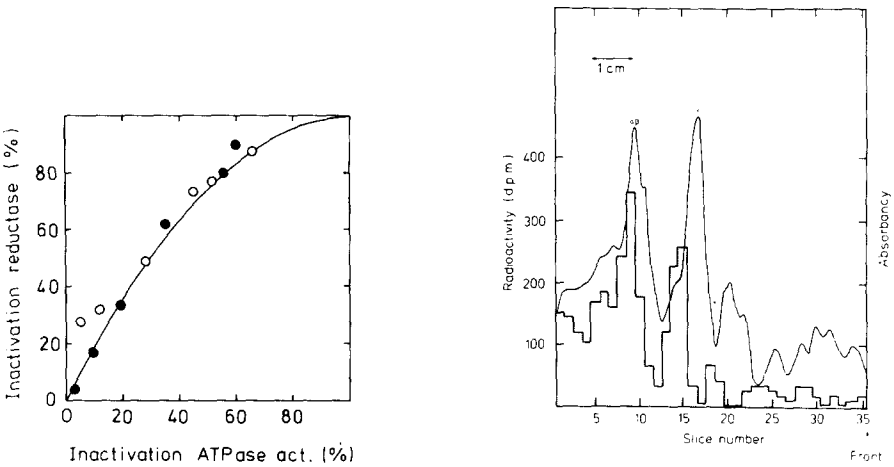

Fig. 5. Comparison of inactivation of the ATP-driven succinate-NAD^+ oxidoreductase with inhibition of ATPase activity after photolabelling of submitochondrial particles with 8-azido-ATP. The results of 2 different experiments are given (○———○, ●———●). The drawn line is a theoretical curve, see text for explanation.

Fig. 6. Polyacrylamide-dodecyl sulphate gel electrophoresis of submitochondrial particles (55 μg) labelled with 8-azido-[2-^{3}H]ATP (19.3 dpm/pmol) for 2 h. The continuous line shows the scanning at 500 nm after Coomassie-blue staining. The radioactivity of the gel is given by the histogram.

Fig. 6 shows that also with particles the $\alpha\beta$ region is labelled, but it is not clear whether it is the α or the β subunit. The amount of bound label was found to be 1.61 ± 0.24 mol label per mol F_1 (assuming that the particles contain 0.56 nmol F_1/mg protein [38]) when the ATPase activity was inactivated by 82%. This result indicates that also in F_1 bound in particles two sites for 8-azido-ATP are present. The radioactivity found at the top of the gel is probably due to aggregated labelled material, which did not enter the gel completely. The radioactivity under the γ band is due to labelling of the adenine nucleotide translocator, which can be labelled with 8-azido-ATP, as shown by Heaton et al. [39], by 8-azido-ADP according to Schäfer et al. [40], and by an arylazido ADP analogue demonstrated by Lauquin et al. [41]. The translocator contains subunits of about the same molecular weight as of the γ subunit [42,43].

Discussion

Photolabelling with 8-azido-ATP and 8-azido-ADP has shown that ATP- and ADP-binding sites are located in the α and/or β subunits in isolated F_1 and in F_1 present the ATPase complex $F_1 \cdot F_0$ and submitochondrial particles. In the absence of Mg^{2+} azido-ATP binds preferably to the β subunits of isolated F_1 and azido-ADP binds to both the α and β subunits. In the presence of Mg^{2+}, azido-ADP binds preferentially to the α subunit. In all cases, ADP and ATP (but not AMP) protect against inactivation. No experiments have been carried out with azido-ATP in the presence of Mg^{2+}, since, under these conditions, it is hydrolysed to azido-ADP. Experiments are planned, however, using azido-[γ-^{32}P,2-^{3}H]ATP.

Whether or not conditions are such that it is the α or β subunit that is

labelled, complete inactivation is obtained when two molecules of the nitreno-analogue are bound, despite the fact that two β subunits and two α subunits are present in the enzyme. For this reason, it was previously suggested that a single molecule of MgATP interacts with both an α and a β subunit and that the split MgADP remains bound to the α subunit [44]. A new model has now been suggested by Slater et al. [45], based on newer data on binding of ADP to F_1, in which it is suggested that the catalytic site for ATP hydrolysis is present on the β subunit and a regulatory anion-binding site is present on the α-subunit. According to this model, ADP (and azido-ADP) in the absence of Mg^{2+}, bind equally well to the α and β subunit whereas ATP binds preferentially to the β subunit. In the presence of Mg^{2+}, however, ADP binds preferentially to the regulatory site.

Fig. 5 shows clearly that the energy-driven reduction of NAD^+ by succinate is considerably more susceptible to photolabelling with azido-ATP than the uncoupled ATPase activity. It has been shown previously with isolated F_1 that inactivation is proportional to the amount of label bound to the protein, showing that there is no interaction between the two ATP-binding sites (two β subunits) [3]. The data in Fig. 5, however, can be explained by assuming that these units do interact during ATP splitting coupled with the reduction of NAD^+ by succinate. If it is assumed that labelling of the ATP binding sites is random and that binding to only one of the two sites is sufficient for inactivation of the coupled reaction, the relationship between the degree of inactivation of the latter and occupation of the ATP binding sites (taken to be equal to the relative inactivation of the ATPase) is given by the line drawn in Fig. 6. The experimental points obtained from two experiments fall close to this line.

This explanation is consistent with the alternating-site mechanism proposed by Kayalar et al. [46]. Since labelling with azido-ATP had no effect on the firmly bound nucleotides [19], it would seem that the latter are not bound to the ATP-binding sites on the β subunit that are accessible to azido-ATP.

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