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PHOTOLABELLING WITH 8-AZIDO-ADENINE NUCLEOTIDES OF ADENINE NUCLEOTIDE-BINDING SITES IN ISOLATED SPINACH CHLOROPLAST ATPase (CF_1)

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Summary

1. Photolabelling of chloroplast ATPase (CF_1) with either 8-azido-ATP or 8-azido-ADP leads to inactivation of the ATPase activity. ATP and ADP protect against the inactivation, whereas AMP does not.

2. Ca^{2+} has little if any effect on the degree of inactivation by photolabelling with 8-azido-ADP, but, at the same degree of inactivation, twice as much label is bound in the presence of Ca^{2+} as in its absence.

3. The degree of inactivation of ATPase and the amount of bound photolabel are independent of the extent of pre-activation of the CF_1.

4. Upon extrapolation to complete inactivation, 2 mol label, either 8-azido-ATP or 8-azido-ADP, are bound per mol enzyme. In the presence of Ca^{2+}, 4 mol 8-azido-ADP can be bound.

5. In all cases the label is bound specifically to the α and β subunits in almost equal amounts. The location of the bound label is not affected by addition of Ca^{2+}, ATP or ADP.

Introduction

The synthesis of ATP in oxidative phosphorylation in bacteria and mitochondria and in photophosphorylation in chloroplasts proceeds in a similar way. The proton gradient built up, either in oxidation processes in the respiratory chain or in photoreactions in chloroplasts and photosynthetic bacteria, is

Abbreviations: F_1, mitochondrial ATPase; CF_1, chloroplast ATPase: F_0, part of ATPase complex embedded in membrane.

used to drive ATP synthesis [1]. Although the molecular mechanism of ATP synthesis has not yet been established [2], it is known that the process is catalysed by the coupling factor complex ($F_1 \cdot F_0$), the protons being led through F_0 embedded in the membrane to F_1, where the synthesis of ATP from ADP and P_i takes place. We are particularly interested in the mechanism of ATP synthesis on F_1. Our approach has been to use photolabelling techniques to characterize adenine nucleotide-binding sites on F_1. Two papers have appeared in which labelling of beef-heart mitochondrial F_1 is described [3,4]. Because of the close resemblance between mitochondrial F_1 and CF_1 we have now applied photolabelling techniques to CF_1.

Isolated CF_1 is, like F_1, cold labile [5] and contains five different subunits [6—8]. In the mitochondria a 6th subunit, the inhibitor protein [9], is bound to F_1. The ξ subunit fulfills this inhibitory role in CF_1 [8]. Thus the ATPase activity of CF_1 is latent. The enzyme can be activated by treatment with trypsin [10], heat [11] or dithiothreitol [12], all of which affect the ξ subunit.

Different adenine nucleotide-binding sites on CF_1 have been reported [13, 14]. It has been suggested on the basis of Ado*PP*[NH]*P* binding that there is one catalytic site and two regulatory sites [13]. Binding of ADP to regulatory sites inactivates the ATPase, while ATP binding activates it [15]. A tightly bound ADP molecule present in isolated CF_1 [15—17] exchanges only very slowly against added ADP, but rapidly against CaATP or MgATP, simultaneously with the hydrolysis of the ATP [18].

Photolabelling studies on CF_1 have been carried out previously with arylazido-ATP, which labels the β subunits, and arylazido-ADP, which labels both the α and β subunits [19]. In this paper photolabelling with 8-azido-ATP and 8-azido-ADP is described.

Methods and Materials

Chloroplast F_1 was isolated from spinach chloroplasts by chloroform extraction and purified according to Lien and Racker [20]. After standing overnight as a precipitate in 55% saturated ammonium sulphate, it was desalted on Sephadex G-50 (16 × 1 cm) in a solution (pH 7.5) containing 250 mM sucrose, 10 mM Tris-H_2SO_4 buffer, 2 mM EDTA and 4 mM ATP, and stored in liquid N_2. If not stated otherwise the enzyme was activated before use by a combined heat and dithiothreitol treatment [20], precipitated in 55% saturated ammonium sulphate, taken up in the above buffer (no ATP) and desalted on Sephadex G-50 in the same buffer.

The protein concentration was measured according to Lowry et al. [21] with bovine serum albumin as standard ($A^{1\%}_{279\,nm} = 6.67$). A molecular weight of 325 000 was assumed [22].

Tightly bound ATP and ADP were determined by extraction according to Harris et al. [23], after desalting 3-times on Sephadex G-50 columns, and measuring the ATP and ADP with luciferin/luciferase [24] as described earlier [4].

The ATPase reaction, carried out at 37°C, was started by addition of about 5 μg CF_1 to a solution (pH 8.0) containing 40 mM Tris-H_2SO_4 buffer, 10 mM ATP and 10 mM $CaCl_2$, and terminated by addition of trichloroacetic acid to 5% (w/v) final concentration. P_i formed was measured according to Sumner

[25]. The method of synthesis of 8-azido-ATP, 8-azido-[2-^{3}H]ATP, 8-azido-ADP, 8-azido-[2-^{3}H]ADP and 8-azido-[β-^{32}P]ADP, as well as the photolabelling procedure and the method to extract radioactively labelled protein from gels to determine the localisation and amount of label bound to protein have been described previously [3,4].

Polyacrylamide-dodecyl sulphate gel electrophoresis was carried out according to Weber et al. [26], using stacking gels as described by Maurer [27].

Results

Fig. 1 shows that CF_1 is inactivated upon irradiation with ultraviolet light (350 nm) in the presence of azido-ATP. ADP protects effectively against this photoinactivation, ATP less effectively, whereas AMP shows no protection. Irradiation in the absence of photoaffinity label has little effect on the activity of CF_1. These results indicate that azido-ATP binds specifically to the protein at the ATP- and ADP-binding sites.

Fig. 2 shows a scan of a polyacrylamide-dodecyl sulphate gel of CF_1 labelled with azido-[2-^{3}H]ATP and the radioactivity of the slices of the gel. All label is located specifically on the α and β subunits, and in almost equal amounts (Table I). Addition of ATP or ADP has no effect on the ratio of label bound to the α and β subunits, only the total amount is diminished. Extrapolation to 100% inactivation yields close to 2 mol label per mol CF_1, indicating at least

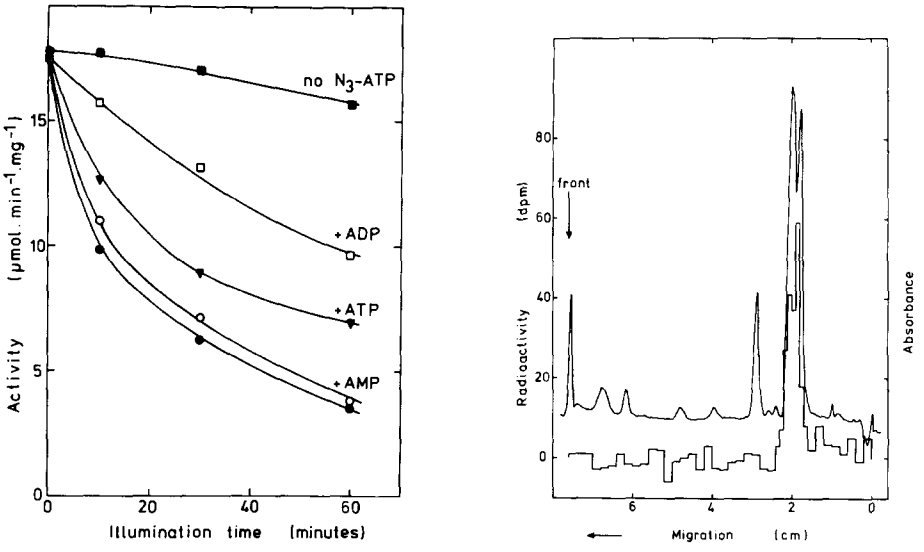

Fig. 1. Photolabelling of isolated CF_1 with 8-azido-ATP (●——●). The illumination dish contained 0.36 mg/ml CF_1, 1.8 mM azido-ATP, 250 mM sucrose, 10 mM Tris-H_2SO_4 buffer and 2 mM EDTA, pH 7.5. Effect of additional 2 mM ATP (▼——▼), 2 mM ADP (□——□) or 2 mM AMP (○——○). Control with azido-ATP omitted (■——■).

Fig. 2. Polyacrylamide-dodecyl sulphate gel electrophoresis of 24.9 μg CF_1 labelled with azido-[2-^{3}H]-ATP (5800 dpm/nmol). The drawn line gives the absorbance at 600 nm of the gel stained by Coomassie brilliant blue. The histogram gives the radioactivity (background subtracted) of the gel slices.

TABLE I

BINDING OF AZIDO-ATP AND AZIDO-ADP TO CF_1 AND THE EFFECT OF Ca^{2+} ON AZIDO-ADP BINDING; AMOUNTS OF TIGHTLY BOUND ADENINE NUCLEOTIDES

CF_1 was labelled with 0.45 mM azido-[2-^{3}H]ATP (45 000 dpm/nmol) or 0.55 mM azido-[2-^{3}H]ADP (125 000 dpm/nmol). The amounts of labelled CF_1 applied to the gels were 30—45 μg. Inactivation percentages were calculated taking the ATPase activity of CF_1 irradiated in the absence of photolabel as 100%. See Figs. 1, 3 and 5 for additional details.

Label	Inactivation (%)	Label bound (mol/mol CF_1)			Tightly bound (mol/mol CF_1)		
		on α	on β	total	ATP	ADP	total
None					0.48	0.76	1.24
8-N_3-ATP	14	0.14	0.19	0.33			
	32	0.21	0.31	0.52			
	60	0.53	0.73	1.26			
	80	0.73	0.77	1.50			
8-N_3-ADP	20	0.15	0.18	0.33			
	41	0.37	0.42	0.79			
	50	0.41	0.49	0.90	0.47	0.92	1.39
	70	0.48	0.63	1.11			
8-N_3-ADP + Ca^{2+} (5 mM)	20	0.35	0.41	0.76			
	53	0.78	0.93	1.71	0.21	0.98	1.19
	82	1.04	1.61	2.65			
	95	1.59	1.82	3.41			

two binding sites for adenine nucleotides on isolated CF_1, one on the α and one on the β subunits.

Fig. 3 shows that, both in the presence and in the absence of Ca^{2+}, CF_1 is also photoinactivated by azido-ADP. The inactivation is diminished by ADP, indicating that azido-ADP binds to the same site(s) as ADP. ATP also protects (not shown) but less effectively, whereas AMP does not. Protection by ADP is more effective in the presence of Ca^{2+}.

Fig. 4 indicates that photolabelling by azido-ADP has no effect on the degree of activation of CF_1 by heat and dithiothreitol.

Table II shows that the amount of bound photolabel is also not affected by the activation of CF_1. These results indicate that the inhibitory function of the ξ subunit is brought about by preventing the ATP splitting on the enzyme and not by preventing adenine nucleotide binding to CF_1, since these sites can be photolabelled also in non-activated CF_1. The distribution of label after photoinactivation with azido-[β-^{32}P]ADP or azido-[2-^{3}H]ATP is also not affected by activation. Ca^{2+} also has no effect on the distribution of label (Table I), although it brings about a doubling of the amount of bound label at the same degree of inactivation of ATPase activity. Addition of ATP or ADP has no effect on the location of the bound azido-ADP as was also found in the case of labelling by azido-ATP.

In Fig. 5 the number of ADP-binding sites on CF_1 that are labelled with azido-ADP is plotted as a function of the degree of inactivation of the ATPase. Without added Ca^{2+} extrapolation to 100% inactivation yields close to 2 mol label per mol CF_1; in the presence of Ca^{2+} twice as much label can be bound.

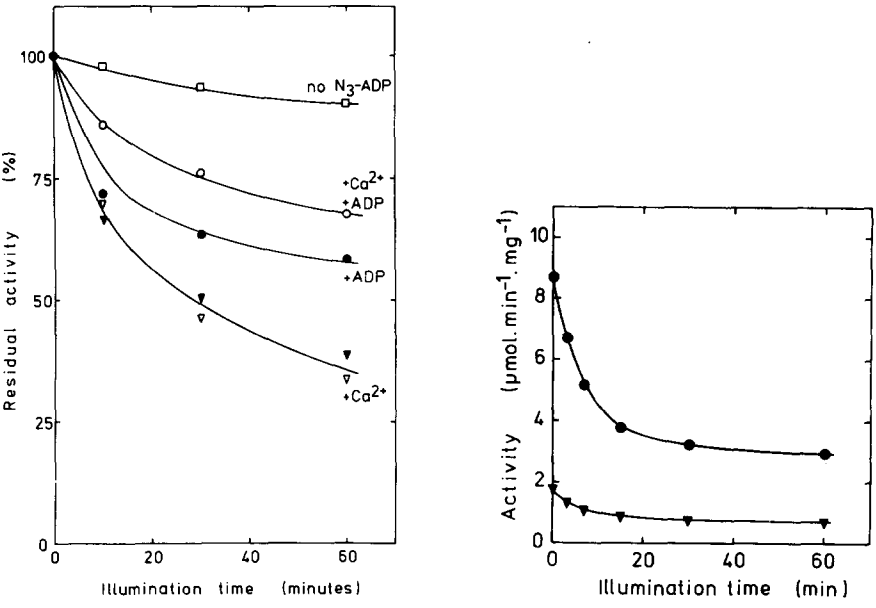

Fig. 3. Photolabelling of isolated CF_1 with 8-azido-ADP in the absence (▼———▼) or presence of Ca^{2+} (▽———▽). The azido-ADP concentration was 1.5 mM and the total Ca^{2+} concentration, added as $CaCl_2$, was 6 mM, or 8 mM when ADP was present; see Fig. 1 for further details. Effect of 2 mM ADP in the absence (●———●) or the presence of Ca^{2+} (○———○) and control with azido-ADP omitted (□———□).

Fig. 4. The activation of CF_1 photolabelled by azido-ADP. Non-activated CF_1 was photolabelled with 1.11 mM azido-ADP for different periods, after which samples were taken and the ATPase activity measured before (▼———▼) and after (●———●) heat and dithiothreitol activation [20].

The equal amounts of label bound to both large subunits in either the absence or the presence of Ca^{2+} (Table I) indicate that both the sites hidden in the absence of Ca^{2+} and those exposed even in the absence of Ca^{2+} have about the same affinity for (azido-)adenine nucleotides. Moreover, in view of the almost equal amounts of label bound on both types of large subunits, two adenine nucleotide-binding sites on both the α and β subunits are likely.

TABLE II

BINDING OF AZIDO-ATP AND AZIDO-ADP TO CF_1 BEFORE AND AFTER ACTIVATION, AND THE ATPase ACTIVITIES OF THE CF_1 PREPARATIONS BEFORE AND AFTER PHOTOLABELLING

CF_1 was labelled by illuminating for 1 h in the presence of 1.94 mM azido-[2-^{3}H]ATP (5800 dpm/nmol) or 1.48 mM azido-[β-^{32}P]ADP (1740 dpm/nmol). See Table I, Fig. 1 and Methods and Materials for additional details.

Label	Preparation	ATPase activity (μmol · min^{-1} · mg^{-1})		Bound label (mol/mol CF_1)
		Before labelling	After labelling	
Azido-ATP	activated	9.4	4.8	1.05
	non-activated	2.4	1.0	1.10
Azido-ADP	activated	11.9	5.1	1.11
	non-activated	4.4	1.6	1.00

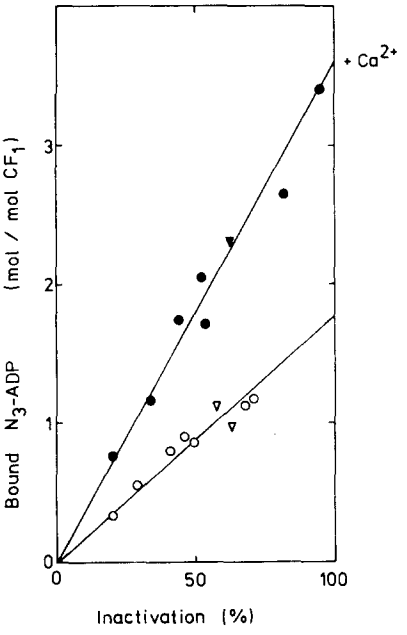

Fig. 5. Azido-ADP bound to CF_1 plotted as a function of the degree of inactivation of the enzyme. CF_1 was labelled for different periods of time with azido-[2-^{3}H]ADP (○———○) or azido-[β-^{32}P]ADP (▽———▽) in the absence of Ca^{2+}, or labelled in the presence of Ca^{2+} with azido-[2-^{3}H]ADP (●———●) or azido-[β-^{32}P]ADP (▼———▼). In the case using azido-[2-^{3}H]ADP as photolabel (0.55 mM), the CF_1 concentration was 5.0 mg/ml instead of 0.36 mg/ml, in order to provide sufficient material for determination of firmly bound nucleotides. See Fig. 3 for further conditions. Labelled CF_1 was subjected to electrophoresis and the radioactivity of the α and β bands was determined. High inactivation percentages (more than 65%) were obtained by removing the photolysed photolabel by gel filtration on a Sephadex G-50 column after 1 h irradiation and addition of new photolabel and prolonged irradiation for varying periods of time.

Table I also shows that the amount of tightly bound nucleotides is little affected by partial photolabelling with azido-ADP in either the absence or the presence of Ca^{2+}.

Discussion

In this paper it is shown that at least four nucleotide-binding sites are present in isolated CF_1, two of which are exposed only when Ca^{2+} is present. Since labelling with azido-ADP in either the absence or the presence of Ca^{2+} has little effect on the amount of tightly bound nucleotides present in isolated CF_1 [16, 17] (Table I), it seems likely that, just as is the case with mitochondrial F_1 [28], two sites for firmly bound nucleotides are present, and that these do not exchange with the azido analogs. Harris et al. [29] have shown that sites for tightly bound nucleotides are much more specific than is the catalytic site for ATP hydrolysis. As was to be expected from their finding that 8-bromo-ADP cannot be exchanged against tightly bound ATP, we have found no exchange at low pH of tightly bound ATP in beef-heart F_1 against azido-ATP. In this light our results suggest that tightly bound ATP and ADP are present in addition to the sites that may be photolabelled with azido-ADP, and that CF_1 contains

more than four sites for adenine nucleotides. This is in analogy with beef-heart F_1 in which, in addition to two regulatory sites on the α subunits and two catalytic sites on the β subunits, sites for tightly bound nucleotides are present [28].

Since the subunit stoichiometry of yeast mitochondrial F_1 is $\alpha_3\beta_3\gamma\delta\epsilon$ [30, 31], which might also be the case for beef-heart mitochondrial F_1, it is possible that the tightly bound nucleotides on this protein are located on the third α and β subunits. In contrast to mitochondrial F_1, a stoichiometry of $\alpha_2\beta_2\gamma\delta\epsilon_2$ is favoured for CF_1 by many authors [32—34], although Yoshida et al. [35] propose $\alpha_3\beta_3\gamma\delta\epsilon$ on the basis of redetermination of the molecular weight of CF_1. If they are correct, the situation might be the same for CF_1 as for mitochondrial F_1. In that case the molecular weight would be 412 500, rather than 325 000 used in the calculations, so that the amounts of bound label in Tables I and II and Fig. 5 should be increased by a factor of 1.27.

CF_1 differs from heart mitochondrial F_1 in that it can be labelled directly with azido-ADP in the presence of Ca^{2+} to the extent of up to 3.4 mol per mol, whereas, in the presence of Mg^{2+}, only 2 mol are covalently bound to mitochondrial F_1. In order covalently to bind about 4 molecules to mitochondrial F_1, successive labelling has to be carried out with azido-ATP in the absence of Mg^{2+} and with azido-ADP in its presence. Reversal of the order of labelling resulted in only 2.8 mol label per mol F_1 being bound. To explain this difference between mitochondrial F_1 and CF_1, we propose that, although in both proteins binding sites for added nucleotide are present on two α and two β subunits, interaction between the α and β subunits, in the absence of Mg^{2+} or Ca^{2+}, prevents binding to more than one site in an $\alpha\beta$ unit. In the presence of Mg^{2+}, binding of ADP or covalent binding of azido-ADP to the α or regulatory sites in mitochondrial F_1 is reflected by strong inhibition of (azido-)ATP binding to the β or catalytic sites [36]. The interaction of the β with the α subunits is broken by the presence of Mg^{2+}, making it possible to label the α subunits even after prelabelling on the β subunits with azido-ATP. In CF_1 the same type of regulation is present when Ca^{2+} is added, as in mitochondrial F_1 in the presence of Mg^{2+}. However, in CF_1 the two regulatory subunits interact with one another. According to Nelson [32], binding of one ADP molecule leads to non-competitive inhibition of ATP binding to the catalytic site, but when two ADP molecules are bound to the regulatory sites binding of ATP to the catalytic sites is facilitated, leading to a higher ATPase activity. This provides a satisfactory explanation of the extra binding of azido-ADP by the addition of Ca^{2+}.

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