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THE NUMBER AND LOCALISATION OF ADENINE NUCLEOTIDE-BINDING SITES IN BEEF-HEART MITOCHONDRIAL ATPase (F_1) DETERMINED BY PHOTOLABELLING WITH 8-AZIDO-ATP AND 8-AZIDO-ADP

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Summary

1. When irradiated 8-azido-ATP becomes covalently bound (as the nitreno compound) to beef-heart mitochondrial ATPase (F_1) as the triphosphate, either in the absence or presence of Mg^{2+}, label covalently bound is not hydrolysed.

2. In the presence of Mg^{2+} the nitreno-ATP is bound to both the α and β subunits, mainly (63%) to the α subunits.

3. After successive photolabelling of F_1 with 8-azido-ATP (no Mg^{2+}) and 8-azido-ADP (with Mg^{2+}) 4 mol label is bound to F_1, 2 mol to the α and 2 mol to the β subunits.

4. When the order of photolabelling is reversed, much less 8-nitreno-ATP is bound to F_1 previously labelled with 8-nitreno-ADP. It is concluded that binding to the α-subunits hinders binding to the β subunits.

5. F_1 that has been photolabelled with up to 4 mol label still contains 2 mol firmly bound adenine nucleotides per mol F_1.

6. It is concluded that at least 6 sites for adenine nucleotides are present in isolated F_1.

Introduction

It is generally accepted that the terminal phosphorylation in the process of oxidative phosphorylation in mitochondria is catalysed by the coupling factor F_1 [1] which also possesses ATPase activity. Similar coupling factors fulfill the

Abbreviations: F_1, mitochondrial ATPase; CF_1, chloroplast ATPase; 8-azido-AXP, both 8-azido-ATP and 8-azido-ADP.

same role in chloroplasts and bacteria.

Kinetic studies [2,3] have shown that mitochondrial F_1 contains both regulatory and catalytic sites for ATP. The catalytic site must necessarily also bind ADP in the ATP synthase reaction. Isolated F_1 contains about 2 mol ATP and 1 mol of ADP.

In previous papers [4,5] we have shown that 8-azido-ATP and 8-azido-ADP may be used as photo-affinity labels for mitochondrial F_1. These compounds have also been used for the enzyme present in bacteria [6,7]. It was shown that the localization of the label depends on the conditions employed. With 8-azido-ATP, practically all the label was found on the β subunit but with 8-azido-ADP it is predominantly on the α subunit when Mg^{2+} is present and about equally on the two types of subunit when Mg^{2+} is absent.

Although based on aurovertin binding to dissociated F_1 [8] we favoured a dimeric structure for heart F_1, it now seems clear that in both bacterial [9—12] and mitochondrial [13,14] F_1 the subunit structure is $\alpha_3\beta_3\gamma\delta\epsilon$. The molecular weight calculated from such a structure and the molecular weights of the isolated subunits [15] is 368 000, which is probably within experimental error of the value (380 000) obtained by sedimentation equilibrium by Yoshida et al. [16].

On the basis of the photo-affinity labelling experiments [4,5] and this subunit structure, it would appear that F_1 contains 6 potential adenine-nucleotide binding sites, one on each of the three α subunits and one on each of the three β subunits. Nevertheless, up to now we have not succeeded in labelling more than two sites. In this paper, we show that if F_1 is first labelled, predominantly on the β subunits, by irradiation in the presence of 8-azido-ATP, followed by irradiation in the presence of 8-azido-ADP and Mg^{2+}, 4 molecules of label, 2 on the α and 2 on the β, are bound. Two of the molecules of adenine nucleotide originally present in the preparation still remain bound, making a total of 6 nucleotide-binding sites.

Materials and Methods

Beef-heart mitochondrial ATPase was isolated according to Knowles and Penefsky [15]. It was stored in liquid N_2 in a buffer (pH 7.5) containing 250 mM sucrose, 10 mM Tris, 4 mM ATP and 2 mM EDTA. Before use the F_1 was filtered on a column containing Sephadex G-50 (coarse) in the same buffer without the ATP. The volume of the column was 10 times that of the F_1 solution. Both prior and subsequent to applying the sample, the column was centrifuged for 1 min at $500 \times g$ (cf. Ref. 17).

F_1 concentrations were determined according to Lowry et al. [18] with bovine serum albumin as standard ($A^{1\%}_{279\,nm} = 6.67$). A molecular weight of 368 000 was assumed. This should be compared with 360 000 assumed in Ref. 4 and 321 000 in Ref. 5.

ATPase activity was measured spectroscopically in an ATP-regenerating system coupled to NADH oxidation (cf. Ref. 4). Bicarbonate (50 mM $KHCO_3$) was present as activating anion [19].

Methods of synthesis of the photolabels 8-azido-ATP, 8-azido-[2-^{3}H]ATP, 8-azido-ADP and 8-azido-[2-^{3}H]ADP and of photolabelling the F_1 have been

described earlier [4,5]. 8-Azido-[γ-^{32}P]ATP was prepared from 8-azido-ATP according to an adaptation of the method of Glynn and Chappell [20] as described by Heaton et al. [21].

Firmly bound nucleotides were extracted from F_1 according to Harris et al. [22] and measured with luciferin/luciferase [23] as described previously [5].

Polyacrylamide gels were prepared from a solution containing 375 mM glycine, 0.5% (w/v) sodium dodecyl sulphate, 0.075% (v/v) *N,N,N',N'*-tetramethylenediamine, 8 M urea, 7% (w/v) acrylamide, 0.7% (w/v) bisacrylamide, adjusted to pH 8.6 with Tris. Polymerisation was initiated by addition of 0.1% (w/v) ammonium persulphate. Stacking gels were used. Radioactivity of bands on the gels were determined as already described [4,5].

Results

Fig. 1 shows the binding of azido-[γ-^{32}P,2-^{3}H]ATP to F_1 in both the presence and absence of Mg^{2+}. In the absence of Mg^{2+}, the binding of azido-ATP is proportional to the inactivation of F_1, extrapolating to about 2 mol label/mol F_1 at complete inactivation (cf. Ref. 4). Since binding of esterified ^{32}P correlated with that of the adenine moiety, measured as ^{3}H, it can be concluded that the terminal phosphate is not appreciably split from the azido-ATP, either bound or free in solution, under these conditions. The free photolabel was hydrolysed to the extent of about 8% after 60 min. In the presence of Mg^{2+}, the label is also initially bound as triphosphate, but after 2 min labelling

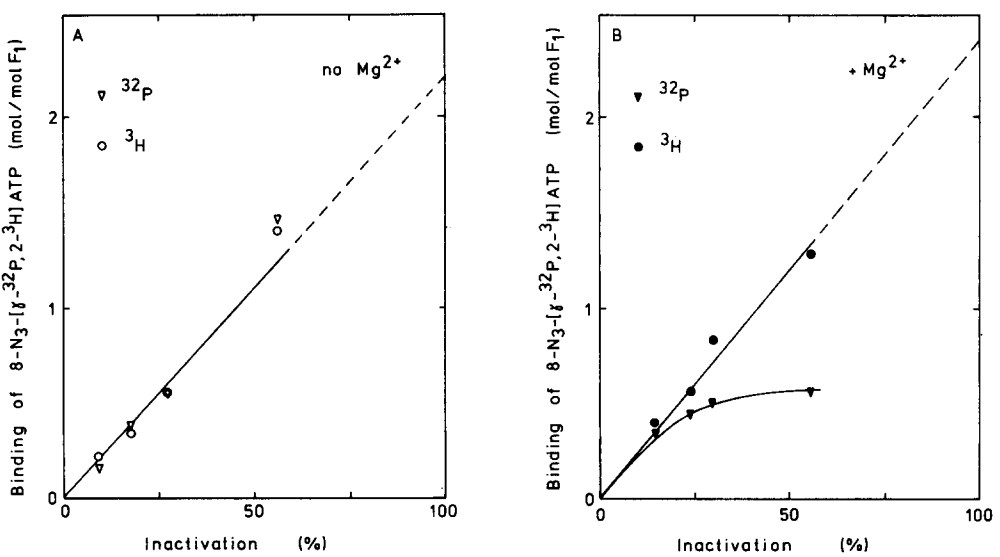

Fig. 1. Bound 8-azido-[γ-^{32}P,2-^{3}H]ATP as function of the inactivation of the F_1-ATPase activity. A, the illumination dish contained 0.58 mM 8-azido-[γ-^{32}P,2-^{3}H]ATP (^{32}P 66 000 dpm/nmol, ^{3}H 38 900 dpm/nmol) and 13.3 mg/ml F_1 in the buffer described in Methods. Samples were taken after 0, 2, 4, 8 and 60 min. B, the illumination dish contained 1.00 mM 8-azido-[γ-^{32}P,2-^{3}H]ATP (^{32}P 23 700 dpm/nmol, ^{3}H 24 200 dpm/nmol), 13.0 mg/ml F_1 and 4 mM $MgCl_2$. Samples were taken after 0, 2, 4, 8 and 16 min. Radioactively labelled F_1 (210—276 μg) was applied to polyacrylamide-urea-dodecyl sulphate gels and subjected to electrophoresis. The gels were extracted directly after electrophoresis and the ^{32}P and ^{3}H were determined [5].

by hydrolysed photolabel (azido-ADP) becomes predominant. After 16 min, 95% of the azido-ATP was hydrolyzed. In this case also the binding is proportional to the inactivation and on extrapolation to complete inactivation rather more than 2 mol label, either azido-ATP or azido-ADP, is bound.

Fig. 2 shows that addition of Mg^{2+} causes binding of azido analogue to the β subunits to diminish, whereas binding to the α subunits is increased. This was also found previously for azido-ADP [2,3].

The results given in Table I show that if labelling is first carried out with azido-ATP, in the absence of Mg^{2+}, followed by azido-ADP in its presence, about 4 molecules of azido analogue become bound, 2 molecules to each subunit. If, however, the order of labelling is reversed, much less label is found in the β subunit (Table I, Expt. 2). Indeed, after prior labelling by azido-ADP, in the presence of Mg^{2+}, only 0.6 mol extra label was incorporated after 1 h incubation with 0.86 mM azido-ATP in the absence of Mg^{2+}, compared with 1.4 mol azido-ATP bound after incubation of untreated F_1 for 1 h with 0.58 mM azido-ATP (see Fig. 1).

Table II shows the effect of covalent binding of the analogues on the amount of ATP and ADP firmly bound to the F_1 [24]. The two experiments refer to the same ones as in Table I. Although the two samples differ appreciably in the amount of ATP present before treatment, the total amount of nucleotide, both covalently bound as the nitreno analogue and non-covalently bound, is about the same in the two cases, namely about 6 mol/mol F_1. Incubation with ATP and ADP of the fully labelled preparation did not result in further binding of nucleotide.

The adenine nucleotides extracted from F_1 after labelling with azido-ATP or

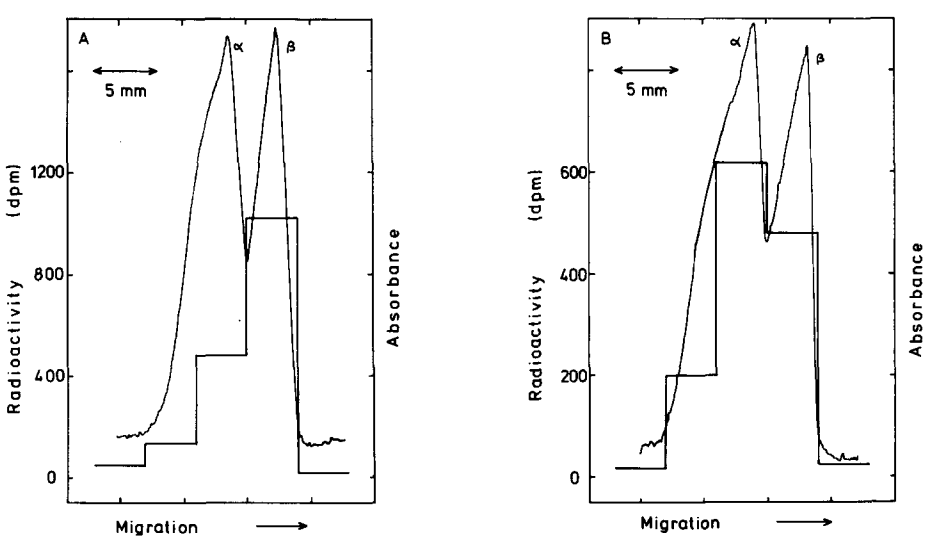

Fig. 2. Polyacrylamide-urea-dodecyl sulphate gel electrophoresis of F_1 photolabelled as indicated in Fig. 1 with 8-azido-[γ-^{32}P,2-^{3}H]ATP for 8 min. A, the labelling was carried out in the absence of Mg^{2+}; 38.8 μg F_1 were applied to the gel. Radioactivity was measured as ^{3}H (38 000 dpm/nmol). B, the labelling was carried out in the presence of Mg^{2+}; 34.5 μg F_1 was applied to the gel. Radioactivity was measured as ^{32}P (38 400 dpm/nmol). The radioactivity is given in the histogram and the line shows the absorbance of the $\alpha\beta$-region of the gel, stained with Coomassie brilliant blue, at 510 nm.

TABLE I

NUMBER AND LOCATION OF BINDING SITES FOR AZIDO-ADENINE NUCLEOTIDES ON ISOLATED F_1

Expt. 1. F_1 (15 mg/ml) was photolabelled with 0.5 mM azido-[2-^{3}H]ATP (12 500 dpm/nmol) in the absence of Mg^{2+} for 1 h. The photolysed label was then removed by gel filtration and the irradiation was continued after addition of new photolabel. This procedure was repeated once, so that a total of 3 h irradiation was achieved. The F_1 preparation obtained in this way (F_1-T, 16 mg/ml) was photolabelled for 1 h with 2.0 mM azido-[2-^{3}H]ADP (13 000 dpm/nmol) in the presence of 6 mM $MgCl_2$ to give the F_1-T-D_{Mg} preparation. Expt. 2. F_1 (30 mg/ml) was labelled with 0.4 mM azido-[2-^{3}H]ATP (23 400 dpm/nmol) in the same way as in Expt. 1. F_1-T (28.6 mg/ml) was further labelled with 0.8 mM azido-[2-^{3}H]-ADP (20 300 dpm/nmol) in the presence of 5 mM $MgCl_2$ for 1 h. After gel filtration, a further amount of azido-ADP was added and the irradiation continued for a further hour, thereby yielding F_1-T-D_{Mg}. F_1-D_{Mg} was obtained by labelling F_1 (15.5 mg/ml) with 0.97 mM azido-[2-^{3}H]ADP (63 900 dpm/nmol) for 1 h in the presence of 5 mM $MgCl_2$. F_1-D_{Mg} (15.7 mg/ml) was further labelled with 0.86 mM azido-[2-^{3}H]ATP (67 600 dpm/nmol) in the absence of Mg^{2+} for 1 h, yielding F_1-D_{Mg}-T. To determine the amount and location of the bound label 20—180 μg labelled F_1 were treated with sodium dodecyl sulphate and subjected to electrophoresis on urea-dodecyl sulphate-polyacrylamide gels. Protein bands were extracted and their radioactivity was measured [4].

Expt.	Preparation	ATPase activity (μmol · min^{-1} · mg^{-1})	Bound label (mol/mol F_1)		
			on α	on β	Total
1	F_1	161			
	F_1-T	28	0.56	1.11	1.67
	F_1-T-D_{Mg}	3.1	2.09	1.51	3.60
			1.53	0.40	1.93
2	F_1	133			
	F_1-T	4.9	0.49	1.20	1.69
	F_1-T-D_{Mg}	1.4	2.12	2.09	4.21
			1.63	0.89	2.52
	F_1-D_{Mg}	22	1.72	0.51	2.24
	F_1-D_{Mg}-T	7.9	1.96	0.89	2.86
			0.24	0.38	0.62

TABLE II

ADENINE NUCLEOTIDE BINDING SITES IN ISOLATED F_1

In two experiments firmly bound nucleotides were measured in F_1 before and after photolabelling as indicated in Table I. The firmly bound nucleotides were extracted by addition of perchloric acid [22]. AdN, adenosine nucleotide.

Expt.	Preparation	Label bound (mol/mol F_1) total	Firmly bound AdN (mol/mol F_1)			Total bound AdN (mol/mol F_1)
			ATP	ADP	total	
1	F_1	—	1.53	1.38	2.91	
	F_1-T	1.67	1.27	1.53	2.80	
	F_1-T-D_{Mg}	3.60	1.08	1.68	2.76	6.36
2	F_1	—	2.41	1.24	3.65	
	F_1-T	1.69	1.04	1.11	2.15	
	F_1-T-D_{Mg} *	4.21	0.74	1.22	1.96	6.17
	F_1-D_{Mg}	2.24	1.13	1.32	2.45	
	F_1-D_{Mg}-T	2.86	1.05	1.37	2.42	5.28

* After incubation for 1 h with 5 mM ATP and 5 mM ADP and removing excess ATP and ADP by eluting 3 times on a Sephadex-50 column, this preparation contained 0.83 mol ATP and 1.11 mol ADP.

azido-ADP contained about 0.2—0.3 mol radioactive nucleotides. After the double labelling (indicated as F_1-T-D_{Mg} or F_1-D_{Mg}-T in Tables I and II) this amount was approximately doubled. This is most likely due to a slow exchange of the firmly bound nucleotides with radioactive ATP and ADP, present to the extent of 1—2% in the azido-ATP and azido-ADP used in these experiments.

Discussion

It has now been shown that it is possible covalently to bind 4 molecules of nitreno-adenine nucleotide to F_1, 2 molecules to the α subunits and 2 molecules to the β subunits. It was shown earlier that binding of only 2 molecules of nitreno-ATP, practically all to the β subunits, is sufficient to inactivate the ATPase completely. Covalent binding of nitreno-ADP to the subunit also leads to inactivation of the ATPase. When the label is predominantly in the α subunit (azido-ADP in the presence of Mg^{2+}), labelling of a total (in both subunits) of rather more than 2 mol, but distinctly less than 3 mol, is required for complete inactivation (see for example Expt. 2, Table I).

It seems likely, then, that binding to only two of the α and β subunits is sufficient for complete inactivation. That somewhat more than 2 molecules are bound in the presence of Mg^{2+} could be due to some non-specific binding (0.03—0.07 mol label is found bound to the γ subunit [5]), but is more likely due to the fact that, under these conditions, some molecules of F_1 are labelled in both the α- and β-subunits (see below). It is significant that, under none of the conditions studied in the present paper, more than two α subunits or more than two β subunits are labelled.

If the α subunits are labelled with azido-ADP, it is not possible fully to label the β subunits by subsequent incubation with azido-ATP (Table I, Expt. 2). Indeed, although nearly as much labelling is found with azido-ADP in the presence of Mg^{2+} after incubation with azido-ATP as without prior incubation, the labelling by azido-ATP in the absence of Mg^{2+} after incubation with azido-ADP in its presence is only about one quarter of that found without prior incubation. A possible explanation is: in the absence of Mg^{2+}, mutual interactions between the α and β subunits prevent binding of more than 1 molecule (azido)-AXP to each $\alpha\beta$-unit. In the presence of Mg^{2+}, the interaction of the α subunit on the β remains, whereas the converse interaction is lost. Under these conditions, the α subunits are regulatory subunits for the β or catalytic subunits (cf. Ref. 25). This provides too a possible explanation of the fact that in the presence of Mg^{2+} somewhat more than 2 mol azido-ADP are bound per mol F_1 (see above).

Chloroplast F_1 differs from mitochondrial F_1 in that, in the presence of activating cation (Ca^{2+}), 4 mol of azido-ADP become bound [26]. We have proposed that this reflects a difference in the nature of the interaction between the subunits.

Even after the prolonged illumination with azido-ATP, then azido-ADP, the mitochondrial F_1 still contains at least two molecules of firmly bound nucleotides. Thus F_1 contains at least 6 and maybe 7 [25] nucleotide-binding sites.

The amounts of ATP and ADP present in isolated F_1 are quite variable (see, for comparison, Ref. 22), as exemplified by the two experiments given in

Table II. This is, in fact, to be expected on the basis of present knowledge of the binding constants of nucleotides to F_1. In Table III, calculations are made for two concentrations of F_1 (50 and 5 μM) suspended in 5 mM ATP and subjected to repeated precipitation with $(NH_4)_2SO_4$. For simplicity, the calculation is made only for ATP and it is assumed that F_1 contains one site to which ATP is bound with a dissociation constant of 10^{-5} μM [27], one with a dissociation constant of 0.5 μM (the value found for ADP by Wielders and Slater [28], two with dissociation constants of 40 μM (cf. Ref. 25) and two with 200 μM (cf. Ref. 25). Assuming independent binding, the amounts of bound ATP shown in Table III may be calculated. It is clear that, after 4 precipitations with ammonium sulphate (the routine procedure), the amount of nucleotide still bound to the so-called weak sites will still be appreciable when 50 μM F_1 is used as starting material and negligible when 5 μM is used. The concentration used in the experiments shown in Tables I and II were, in fact, 6.9 μM and 48.9 μM, respectively. Thus, the loss of bound nucleotide found after labelling in Exp. 2, approx. 1.5 mol/mol F_1, can be accounted for by loss of so-called loosely bound nucleotide.

Esch and Allison [13] were able to attach 3 molecules of the affinity label *p*-fluorosulfonyl-benzoyl-5'adenosine to the β subunit of F_1 after the latter was treated with glycerol to remove firmly bound nucleotide. It seems likely, then, that one of the firmly bound nucleotides is bound to the β subunit. It is, perhaps, not surprising that this nucleotide is not replaced by the azido analogue

TABLE III

'FIRMLY' AND 'LOOSELY' BOUND ATP OF ISOLATED F_1, CALCULATED AFTER 1—6 STEPS TO REMOVE FREE ATP BY AMMONIUM SULPHATE-PRECIPITATION

The calculations are based on the usual method to remove free ATP from F_1, i.e. addition of saturated ammonium sulphate to half saturation, centrifuging the precipitated F_1, dissolving the pellet in buffer, again adding ammonium sulphate and repeating the procedure until in total 4 precipitations are carried out [22]. Calculations are shown for the ATP that is firmly bound (to the sites with $K_d = 10^{-5}$ and 0.5 μM), loosely bound (to the sites with $K_d = 40$ and 200 μM) and free in the pellet after 1—6 precipitation steps. The following assumptions are made: (i) the volume of the pellet, containing free ATP, is estimated to be 6 μl/mg F_1, (ii) the K_d values are not affected by the ammonium sulphate, (iii) and the $F_1 \cdot$ ATP equilibrium is established after the addition of the ammonium sulphate.

F_1 (μM)	Ammonium sulphate precipitations (number)	(mol/mol F_1)			
		Tightly bound ATP	Loosely bound ATP	Free ATP	Total ATP
50	1	2.00	3.81	5.09	10.90
	2	2.00	2.49	0.35	4.83
	3	1.99	1.30	0.08	3.38
	4	1.97	0.74	0.04	2.75
	5	1.95	0.44	0.02	2.41
	6	1.91	0.28	0.01	2.20
5	1	2.00	3.82	5.37	11.19
	2	1.98	0.87	0.04	2.89
	3	1.82	0.13	0.00	1.95
	4	1.61	0.05	0.00	1.66
	5	1.46	0.03	0.00	1.49
	6	1.36	0.02	0.00	1.38

since Harris et al. [29] have shown that the closely related compound, 8-bromo-ADP, cannot exchange with the firmly bound ATP under conditions in which ADP does so. Preliminary experiments, using the method described by Harris et al. [29], indicate that 8-azido-ADP behaves similarly to 8-bromo-ADP.

In view of the subunit structure $\alpha_3\beta_3\gamma\delta\epsilon$, it is, in fact, attractive to assume that the 6 adenine-nucleotide binding sites identified in this paper are located on each of the α and the β subunits. The fact still has to be explained, however, why one of each α and β subunits binds adenine nucleotide so much more strongly and the azido nucleotide (and aurovertin and *N,N'*-dicyclohexylcarbodiimide [30]) so much more weakly than the other α and β subunits. It would be interesting to know which of the three β subunits is bound by 4-chloro-7-nitrobenzofurazan when the enzyme is inactivated [31].

In fact, despite the strong evidence in favour of the structure $\alpha_3\beta_3\gamma\delta\epsilon$, F_1 behaves catalytically like a protein with an $\alpha_2\beta_2$-structure.

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