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SPECIFIC LABELLING OF THE (Ca^{2+} + Mg^{2+})- ATPase OF *ESCHERICHIA COLI* WITH 8-AZIDO-ATP AND 4-CHLORO-7-NITROBENZOFURAZAN

J.H. VERHEIJEN, P.W. POSTMA and K. VAN DAM

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

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Summary

1. 8-Azido-ATP is a substrate for *Escherichia coli* (Ca^{2+} + Mg^{2+})-ATPase (*E. coli* F_1).

2. Illumination of *E. coli* F_1 in the presence of 8-azido-ATP causes inhibition of ATPase activity. The presence of ATP during illumination prevents inhibition.

3. 8-Azido-ATP and 4-chloro-7-nitrobenzofurazan (NbfCl) bind predominantly to the α subunit of the enzyme, but also significantly to the β subunit.

4. The α subunit of *E. coli* F_1 seems to have some properties that in other F_1-ATPases are associated with the β subunit.

Introduction

The subunit composition and catalytic properties of ATPases (in this paper called F_1-ATPases, irrespective of the source of the enzyme) isolated from various energy-transducing membranes are strikingly similar [1—4]. They usually seem to contain five types of subunits, named α, β, γ, δ and ϵ according to their mobility during sodium dodecyl sulphate polyacrylamide gel electrophoresis. The stoicheiometry of the subunits is still a matter of debate. According to some authors it is $\alpha_3\beta_3\gamma\delta\epsilon$, but others find $\alpha_2\beta_2\gamma_2\delta_x\epsilon_y$ with x and y probably 1 or 2 [1—6]. It is not certain, however, that the stoicheiometry of the subunits of the F_1-ATPase is the same in all organisms. Despite this similarity between the various ATPases, there are some differences. The molecular weights of the analogous subunits from different organisms are not completely identical [1—4]. In some F_1-ATPases the inhibitory subunit is one of the five subunits [1—3,7], whereas in other F_1-ATPases a sixth polypeptide is present

Abbreviations: F_1, ATPase from various energy-transducing membranes; NbfCl, 4-chloro-7-nitrobenzofurazan.

which is the protein inhibitor originally isolated by Pullman and Monroy [8]. The recently described F_1 from the thermophilic bacterium PS3 seems to have no protein inhibitor [9]. The sensitivity to different inhibitors is not the same for all F_1-ATPases (for review see ref. 3). Because of these similarities and differences it is interesting to compare the properties of the analogous ATPase subunits from different organisms. In all F_1-ATPases studied until now, the ATPase activity is associated with the α or β subunits or maybe with a complex of both subunits [1,9].

The properties of F_1-ATPases, in particular the various adenine nucleotide-binding sites, can be studied now with a number of inhibitors that bind covalently to F_1-ATPase from beef heart and other sources. The photoaffinity label 8-azido-ATP was used by Wagenvoord et al. [5] to inhibit and label the ATPase from beef heart mitochondria. This compound binds covalently and specifically to the β subunit of the enzyme [5]. Ferguson et al. [10,11] used 4-chloro-7-nitrobenzofurazan (NbfCl) as an inhibitor of heart mitochondrial ATPase and showed that it also binds covalently to the β subunit of the enzyme [10,11]. Deters et al. [12] and Nelson et al. [13] showed that the same inhibitor binds to the β subunit of the ATPase of chloroplasts and *Escherichia coli*, respectively. In this paper it will be shown that *E. coli* F_1-ATPase is inhibited irreversibly by 8-azido-ATP after illumination at 350 nm. In contrast to beef heart F_1, however, 8-azido-ATP as well as NbfCl bind primarily covalently to the α subunit of the *E. coli* enzyme.

Materials and Methods

Growth of bacteria. *E. coli* K12 cells, strain 1100 (obtained from R.D. Simoni), were grown aerobically in 15-l cultures in a minimal salt medium containing 7 g K_2HPO_4, 3 g KH_2PO_4, 0.1 g $MgSO_4$ and 2.0 g $(NH_4)_2SO_4$ per l supplemented with 1 mg/l thiamine and 0.5% (w/v) glucose as a carbon source. Stationary phase cells were harvested and washed twice with 50 mM Tris · HCl/2.5 mM $MgCl_2$ (pH 7.5). Membranes were prepared by passing cells through a French pressure cell at a pressure of 1200 kg/cm^2.

Isolation of F_1-ATPase. After washing membranes with a low ionic strength buffer, F_1-ATPase was purified as described by Futai et al. [14]. Protein was determined according to Lowry et al. [15].

ATPase assays. ATPase activity was determined by measuring inorganic phosphate liberated from ATP after incubating at 37°C for 5—15 min [16]. The incubation mixture, final volume 0.20 ml, contained 50 mM Tris · HCl, 5 mM ATP, 5 mM phosphoenolpyruvate, 2.5 mM $MgCl_2$ and 30 μg/ml (6 units/ml) pyruvate kinase, pH 8.0. In some experiments ATPase activity was measured by coupling the reaction to NADH oxidation at 25°C in the following medium: 50 mM Tris · HCl/5 mM ATP/0.5 mM phospho*enol*pyruvate/2.5 mM $MgCl_2$/0.15 mM NADH/15 μg/ml (3 units/ml) pyruvate kinase/7.5 μg/ml (4 units/ml) lactate dehydrogenase, final pH 8.0.

Gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) using 7.5 or 12% acrylamide gels was carried out in a discontinuous system as described by Laemmli [17]. The gels were stained for 2 h with 0.04% (w/v) Coomassie Brilliant Blue in 30% (v/v) meth-

anol/10% (v/v) acetic acid. Gels were destained in 30% (v/v) methanol/10% (v/v) acetic acid, and scanned at 500—600 nm in a Zeiss (ZK 4) gel scanner. To determine the distribution of radioactivity in the gels, gels, previously stained with Coomassie Brilliant Blue, were frozen in solid CO_2 and sliced in 1- or 2-mm slices with a gel slicer (Mickle). Slices were digested in 0.5 ml Protosol (New England Nuclear)/water (9 : 1, v/v) and were counted in a scintillation mixture containing 5 g PPO and 0.3 g POPOP per l of toluene.

Inhibition and labelling by 8-azido-ATP. Inhibition and labelling was studied in the following way. A small volume of 50 mM Tris · HCl (pH 8.0)/0.5 mM EDTA containing particles or F_1 and 8-azido-ATP was illuminated with ultraviolet light (350 nm, CAMAG, Type TL 900, 8W lamp) at room temperature or 4°C.

Inhibition and labelling by NbfCl. Inhibition and labelling by NbfCl was carried out as follows. Particles or F_1 in 50 mM Tris · HCl (pH 8.0)/0.5 mM EDTA were treated with various concentrations of NbfCl for different times, at room temperature. To prevent binding of labelled NbfCl to the denatured subunits in sodium dodecyl sulphate we added a ten times higher amount of unlabelled NbfCl before samples were dissolved in sodium dodecyl sulphate.

Materials. NbfCl was a product of Pierce and [U-^{14}C]NbfCl was obtained from Centre d'Études Nucleaires de Saclay, Service des Molécules Marquées (Gif-sur-Yvette, France) and diluted to 44 Ci/mol. 8-Azido-ATP and 8-azido-[2-^{3}H]ATP (56 Ci/mol) were generous gifts of R.J. Wagenvoord. All other chemicals were obtained from commercial sources.

Results and Discussion

Influence of 8-azido-ATP on ATPase activity of particles and purified F_1

To determine whether 8-azido-ATP can be used as a specific active site-directed inhibitor of *E. coli* F_1, membrane particles were illuminated in the presence of 8-azido-ATP. After dilution of the membranes (20—40-fold) the remaining ATPase activity was measured. Table I shows that a light-dependent inhibition of ATPase activity by 8-azido ATP occurs which can be prevented by having ATP present during the illumination period. A similar experiment with

TABLE I

INFLUENCE OF 8-AZIDO-ATP ON ATPase ACTIVITY OF MEMBRANE PARTICLES

0.20-mg particles in a volume of 0.1 ml 50 mM Tris · HCl (pH 8.0)/0.5 mM EDTA plus additions as indicated, were illuminated for 30 min with ultraviolet light at 4°C as described in Materials and Methods. Before and after illumination samples were taken and ATPase activity was determined as described in Materials and Methods.

Additions (present during illumination)	ATPase activity (μmol · min^{-1} · mg^{-1})	
	Before illumination	After illumination
No addition	0.62 (100%)	0.56 (90%)
4 mM 8-azido-ATP	0.59 (95%)	0.13 (21%)
4 mM ATP	0.61 (98%)	0.66 (106%)
4 mM 8-azido-ATP plus 4 mM ATP	0.63 (102%)	0.61 (98%)

TABLE II

INFLUENCE OF 8-AZIDO-ATP ON ATPase ACTIVITY OF PURIFIED F_1

Purified F_1 (1.5 μg) in a volume of 50 μl 50 mM Tris · HCl (pH 8.0)/0.1 mM EDTA/0.5 mg/ml bovine serum albumin plus additions, was illuminated with ultraviolet light for 60 min at room temperature. Before and after illumination samples were taken for determination of ATPase activity as described in Materials and Methods.

Additions (present during illumination)	ATPase activity (μmol · min^{-1} · mg^{-1})	
	Before illumination	After illumination
No additions	33.0 (100%)	33.6 (102%)
4 mM 8-azido-ATP	32.7 (99%)	2.9 (9%)
4 mM ATP	37.2 (113%)	39.1 (118%)
4 mM 8-azido-ATP plus 4 mM ATP	35.6 (108%)	24.1 (73%)
4 mM ADP	38.0 (115%)	34.2 (104%)
4 mM ADP plus 4 mM 8-azido-ATP	36.5 (111%)	28.0 (85%)

purified F_1 is shown in Table II. In this case bovine serum albumin was added during illumination with ultraviolet light to protect F_1, since it was found that without serum albumin at the low protein concentrations used the ATPase activity decreased about 45%. Table II shows that both ATP and ADP prevented inhibition.

To show that 8-azido-ATP is at least interacting with the active ATP-splitting site, we determined whether 8-azido-ATP could be hydrolysed by the enzyme when incubated in the dark. In Fig. 1A it is shown that 8-azido-ATP is hydrolysed by *E. coli* F_1 with Michaelis-Menten kinetics. The V of our enzyme preparation for 8-azido-ATP is about 30-fold lower than that for ATP, determined under the same conditions (Fig. 1B) similar to results obtained with the beef heart enzyme [5]. The K_m of the *E. coli* enzyme for 8-azido-ATP is somewhat higher than for ATP, but the difference is less than in the case of beef heart F_1

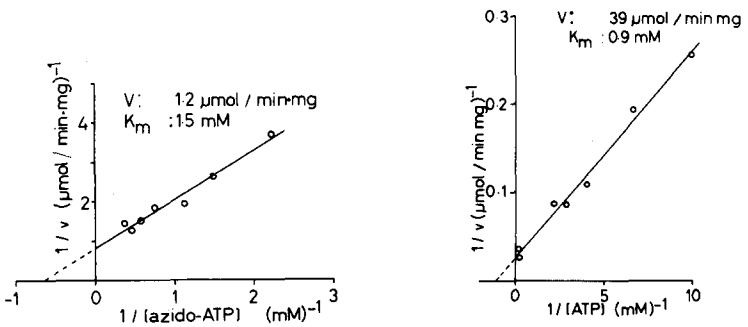

Fig. 1. (A) Hydrolysis of 8-azido-ATP by *E. coli* F_1. 12 μg of F_1 was incubated in 2.0 ml incubation mixture at 25°C with various concentrations of 8-azido-ATP. Enzyme activity was determined by measuring NADH oxidation as described in Materials and Methods. Because pyruvate kinase has a rather low affinity for 8-azido-ADP, a ten times higher amount of the enzyme was used. (B) Hydrolysis of ATP by *E. coli* F_1. 0.6 or 1.2 μg of F_1 was incubated in 2.0 ml of incubation mixture at 25°C with various concentrations of ATP. Enzyme activity was determined by measuring NADH oxidation as described in Materials and Methods.

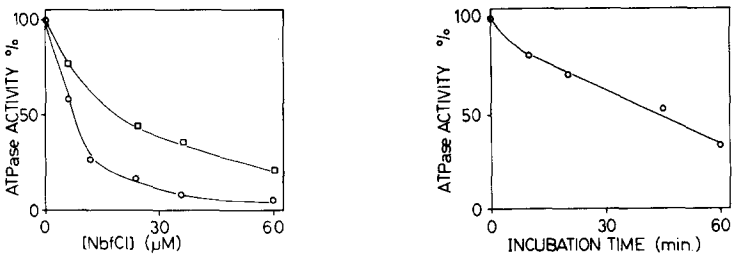

Fig. 2. Inhibition of ATPase activity by NbfCl. (A) Influence of NbfCl on ATPase activity of particles and F_1. 3 μg of F_1 (○———○) or 20-μg particles (□———□) were incubated for 30 min at 4°C in 160 μl 50 mM Tris · HCl (pH 8.0) and various amounts of NbfCl. (B) Time dependence of NbfCl inhibition of F_1. 3 μg of F_1 was incubated for various times at 4°C in 160 μl 50 mM Tris · HCl (pH 8.0) with 10 μM NbfCl. ATPase activity was determined by measuring liberated inorganic phosphate as described in Materials and Methods. Control experiments show that incubation in the absence of NbfCl has no influence on the ATPase activity.

[5]. Experiments with 8-azido-ADP show that 5 mM 8-azido-ADP inhibited the ATPase acitivity only 15—20% under similar conditions as used with 8-azido-ATP. In binding experiments with labelled 8-azido-ADP almost no binding to F_1 was observed.

Influence of NbfCl on ATPase activity of particles and purified F_1

Ferguson et al. [10,11] have used NbfCl as an inhibitor of beef heart F_1. They showed that after binding one molecule of NbfCl to the β subunit of F_1, the ATPase activity was almost completely inhibited. Fig. 2A shows that the ATPase activity of particles and purified F_1 of *E. coli* can be inhibited by relatively low concentrations of NbfCl. Moreover, a relatively short incubation time is required to inhibit F_1-ATPase by this compound (Fig. 2B). Nelson et al. [13] also found inhibition of *E. coli* F_1 by the same concentration of NbfCl (10 μM), but they used incubation times up to 24 h.

Radioactive labelling of purified ATPase by 8-azido-ATP and NbfCl

To determine which of the subunits binds 8-azido-[2-^{3}H]ATP and [U-^{14}C]-NbfCl, purified F_1 was treated with these inhibitors and examined by polyacrylamide gel electrophoresis as described in Materials and Methods. Fig. 3, A and B show that in both cases most of the label can be found in the α, β region of the gel and a small amount in the γ region. In contrast to the F_1 preparation described by Futai et al. [14], our ATPase preparation contains almost no δ subunit, which is most likely lost during purification of F_1. This might be due to the *E. coli* strain used, because our procedure was identical to the one used by Futai et al. [14]. The ε subunit can hardly be seen on some of the gel scans due to the low amount of protein that was used in order to obtain a better separation of the α and β subunits. A total separation of the α and β subunits could be obtained by using polyacrylamide gels with a lower acrylamide percentage. In this low gel percentage, however, the small subunits cannot be resolved and move at or before the front marker. The α and β subunits behave normally during gel electrophoresis in that their order is similar on 7.5 and 12% acrylamide gels (compare Figs. 3 and 4). It is shown in Figs. 4A, 4B and

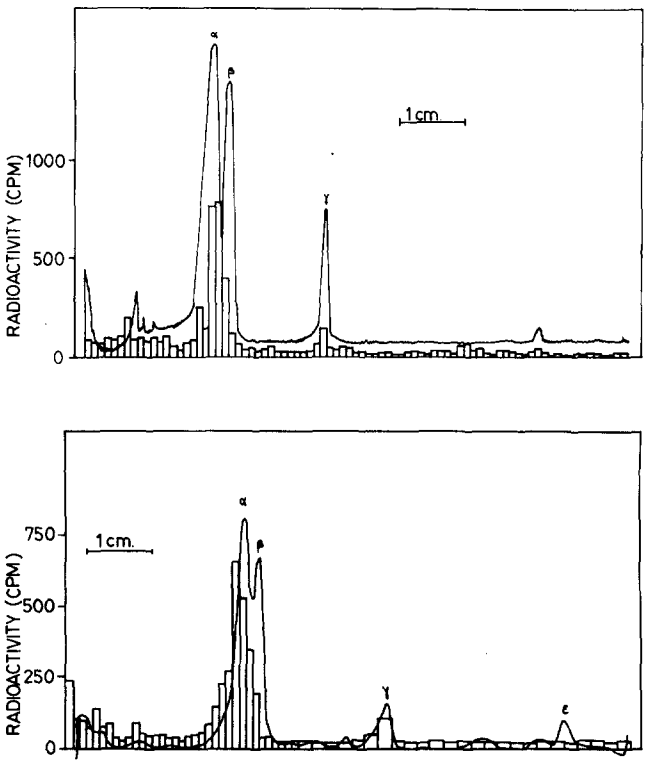

Fig. 3. (A) Gel electrophoresis of 8-azido-[2-^{3}H]ATP-labelled F_1. 30 μg of F_1 treated with 2 mM 8-azido-[2-^{3}H]ATP ($3.2 \cdot 10^4$ cpm/nmol) for 2 h at 4°C as described, was denatured in sodium dodecyl sulphate and applied to a 12% polyacrylamide gel. Gels were stacked at a current of 1 mA/gel and run at 3 mA/gel. Labelling, gel electrophoresis and radioactivity measurements were done as described in Materials and Methods. The bars indicate radioactivity, the solid line indicates absorbance at 500—600 nm of the same gel. (B) Gel electrophoresis of [U-^{14}C]NbfCl-labelled F_1. 14 μg of F_1 was treated for 30 min with 10 μM [U-^{14}C]NbfCl ($7.9 \cdot 10^4$ cpm/nmol) at room temperature. After the incubation, a 10-fold excess of unlabelled NbfCl was added before the sample was treated with sodium dodecyl sulphate. During this treatment no 2-mercaptoethanol was present. Electrophoresis was done on a 12% gel. Further details were as in A.

Table III that both 8-azido-ATP and NbfCl are bound predominantly to the α subunit of the enzyme, although some label is found on the β subunit. From the data in Table II and Fig. 4A it can be calculated that the binding of 1 mol of 8-azido-ATP per mol of F_1 results in an inhibition of more than 90%. Since only about 25% of 8-azido-ATP is bound to the β subunit these results suggest that inhibition of the ATPase activity is not the result of binding of 8-azido-ATP to the β subunit. The findings with NbfCl are less conclusive since from Figs. 2B and 4B it can be calculated that the binding of 0.8 mol NbfCl per mol of F_1 to the α subunit and 0.4 mol NbfCl per mol of F_1 to the β subunit results in about 40% inhibition. It is possible in principle that complete inhibition of the ATPase is caused by binding of 1 mol of NbfCl per mol of F_1 to one β subunit. Different results have been obtained with beef heart F_1 by Wagenvoord et al. [5] with 8-azido-ATP and by Ferguson et al. [10,11] with NbfCl. These authors find that 8-azido-ATP and NbfCl are associated almost exclusively with

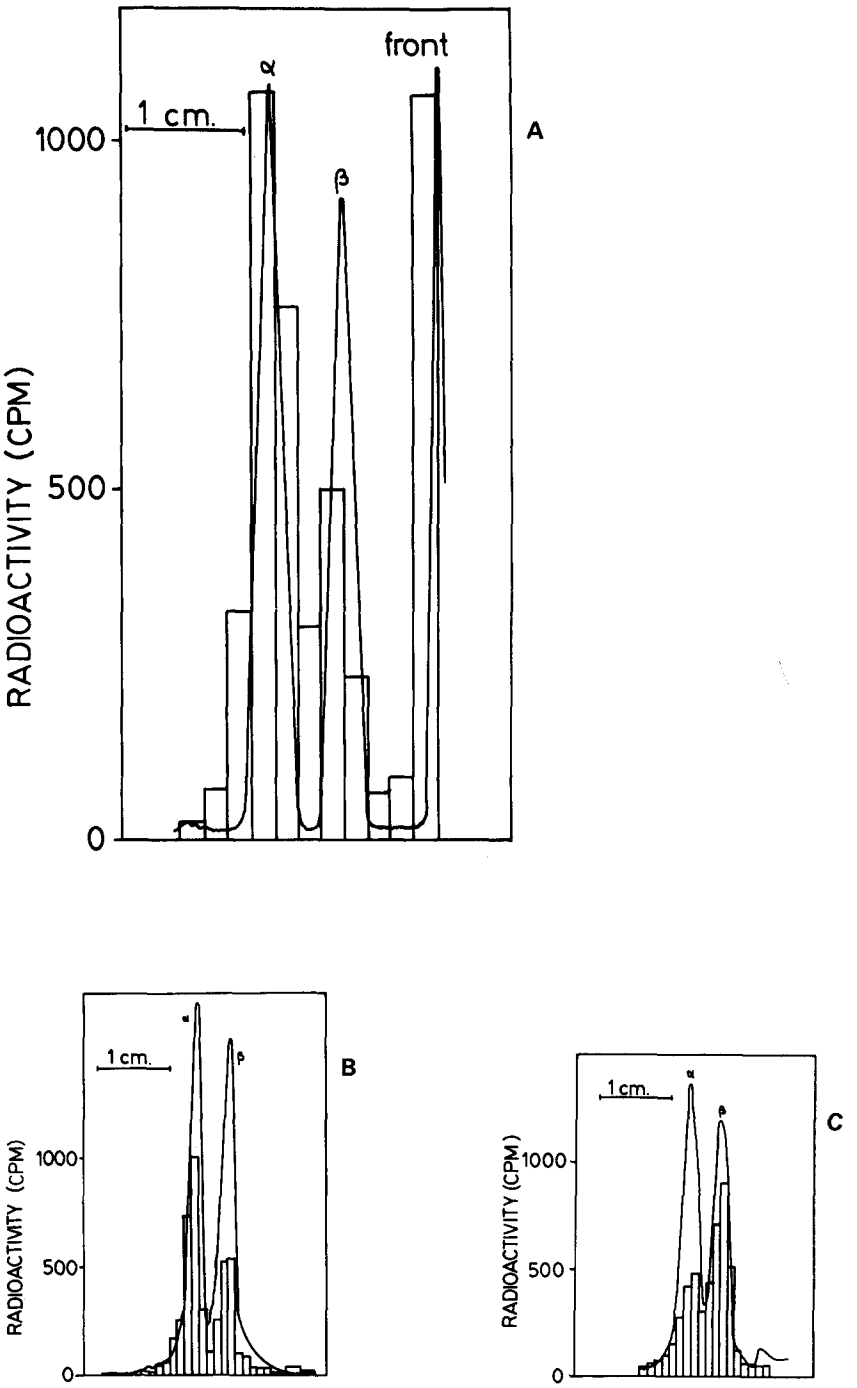

Fig. 4. (A) Gel electrophoresis of 8-azido[2-^{3}H]ATP-labelled F_1. 30 μg of F_1 treated with 2 mM 8-azido-[2-^{3}H]ATP was applied to a 7.5% polyacrylamide gel. Further details were as described in the legend of Fig. 3A. (B) Gel electrophoresis of [U-^{14}C]NbfCl-labelled F_1. The same experiment as described in Fig. 3B was done. The sample was, however, applied to a 7.5% polyacrylamide gel. (C) Gel electrophoresis of [U-^{14}C]NbfCl-labelled F_1. Experimental conditions were as in B except that the incubation was 24 h instead of 30 min.

TABLE III

BINDING OF NbfCl AND 8-AZIDO-ATP TO THE α AND β SUBUNITS

Data are taken from Figs. 4A, 4B and 4C.

Inhibitor used	Percentage of radioactivity in	
	α	β
8-Azido-ATP	72	28
NbfCl, 30 min incubation	64	36
NbfCl, 24 h incubation	34	66

the β subunit of the enzyme. It must be remarked that NbfCl binding as observed by Ferguson is first with a tyrosine residue with an unknown place on the enzyme and is shifted to a nitrogen residue on the β subunit after a pH treatment. Moreover, Deters et al. [12] and Nelson et al. [13] found that NbfCl binds mainly to the β subunit of the enzyme from chloroplasts and from *E. coli*. In the case of *E. coli*, however, they used very long incubation times which might result in aspecific labelling of the subunits. To test this possibility we also analysed F_1-ATPase by gel electrophoresis after a 24 h incubation with [^{14}C]NbfCl. Under those conditions we also find most of the radioactivity in the β subunit (Fig. 4C).

Conclusions

The results presented in this paper indicate that 8-azido-ATP is a photoaffinity label for *E. coli* F_1 and that it binds covalently primarily to the α subunit of the enzyme. It can be concluded from our results that the α subunit contains one or more nucleotide-binding sites, essential for enzyme activity. Since 8-azido-ATP is also a substrate for F_1, it is possible that the labelled nucleotide binding sites are part of the active ATP-splitting site of the enzyme. Whereas ADP is an inhibitor of the ATPase and ADP also protects F_1 against inhibition by 8-azido-ATP (Table II), it is remarkable that 8-azido-ADP has a very low affinity for *E. coli* F_1. The beef-heart enzyme, however, is readily inhibited by 8-azido-ADP and labelled 8-azido-ADP becomes covalently attached to the enzyme (Wagenvoord, R.J., personal communication).

The experiments with the highly specific photoaffinity label 8-azido-ATP combined with the experiments done with the much less specific inhibitor NbfCl show that the α subunit of the *E. coli* enzyme has at least some of the properties that in F_1-ATPases from other organisms are associated with the β subunit. It must be remarked, however, that the subunit nomenclature is in all cases based exclusively on the mobility on SDS polyacrylamide gels and has no functional justification.

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References

1 Kozlov, V.A., Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29—89
2 Senior, A.E. (1973) Biochim. Biophys. Acta 301, 249—277
3 Abrams, A., Smith, J.B. (1974) in The Enzymes (Boyer, P.D., ed.), Vol. X, pp. 395—429, Academic Press, New York
4 Kagawa, Y., Sone, N., Yoshida, M., Hirata, H. and Okamoto, H. (1976) J. Biochem. 80, 141—151
5 Wagenvoord, R.J., van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17—24
6 Verschoor, G.J., van der Sluis, P.R. and Slater, E.C. (1977) Biochim. Biophys. Acta, in the press
7 Nieuwenhuis, F.J.R.M. and Bakkenist, A.R.J. (1977) Biochim. Biophys. Acta 459, 596—604
8 Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762—3769
9 Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480—3485
10 Ferguson, S.J., Lloyd, W.J., Lyons, M.H. and Radda, G.K. (1975) Eur. J. Biochem. 54, 117—126
11 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1975) Eur. J. Biochem. 54, 127—133
12 Deters, D.W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041—1047
13 Nelson, N., Kanner, B.I. and Gutnick, D.L. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2720—2724
14 Futai, M., Sternweis, P.C. and Heppel, L.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2725—2729
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Fiske, C.H. and Subba Row, Y. (1925) J. Biol. Chem. 66, 375—400
17 Laemmli, U.K. (1970) Nature 227, 680—685