Evidence that 4-azido-2-nitrophenylphosphate binds to the phosphate site on the β-subunit of *Escherichia coli* BF_1-ATPase

Richard Pougeois, Guy J.-M. Lauquin and Pierre V. Vignais

Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex and *Faculté de Médecine de Grenoble, France*

Received 13 December 1982

The P_i binding site on bacterial ATPases, isolated from *Escherichia coli* and from the thermophilic bacterium PS3, was located by means of radiolabeled 4-azido-2-nitrophenylphosphate (ANPP), a photoaffinity derivative of P_i. Complete inactivation of both ATPases required 1 mol [^{32}P]ANPP/mol ATPase. Only the β-subunit was photolabeled.

Bacterial ATPase *Phosphate binding site* *Photoaffinity* *4-Azido-2-nitrophenylphosphate*

1. INTRODUCTION

The purpose of the present work was to localize the P_i binding site on purified bacterial ATPase (BF_1). We have reported that ANPP, a photoreactive analogue of P_i, labels the β-subunit of MF_1 and that the binding of 1 mol ANPP fully inactivates MF_1 [1]. Here, we show unambiguously that the P_i binding site of BF_1 is located on the β-subunit. We also present data on photolabeling of TF_1, a thermostable ATPase isolated from the thermophilic bacterium PS3 [2].

2. MATERIALS AND METHODS

2.1. *Materials*

[^{14}C]DCCD (54.5 Ci/mol) and carrier-free $^{32}P_i$ were purchased from the Commissariat à l'Energie Atomique (Saclay), EN^{3}HANCE and [^{14}C]NEM (40 Ci/mol) from New England Nuclear. ATP, ADP, phosphoenolpyruvate and pyruvate kinase (in 50% glycerol) were obtained from Boehringer. ANPP and [^{32}P]ANPP were synthesized and purified as in [1]. All other chemicals were of analytical grade. BF_1 from *E. coli* AN 180 was purified essentially as in [3] and stored as an ammonium sulfate precipitate in 2 M ammonium sulfate, 20 mM Tris–HCl, 1 mM EDTA, 0.5 mM ATP (pH 7.5) at 4°C. TF_1 from PS3 [2] was a gift from Professor Kagawa. *E. coli* cells were generously given to us by the Mérieux Institute (Lyon).

Abbreviations: P_i, inorganic phosphate; MF_1, mitochondrial ATPase; BF_1, *E. coli* ATPase; TF_1, ATPase isolated from the thermophilic bacterium PS3; CF_1, chloroplast F_1; ANP, 4-azido-2-nitrophenol; ANPP, 4-azido-2-nitrophenylphosphate; TMMg, 50 mM Tris, 50 mM MES, 1 mM $MgSO_4$ (pH 7.5); TMES, 50 mM Tris, 50 mM MES, 1 mM EDTA, 1 mM Na_2SO_4 (pH 7.5); NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate

2.2. *ATPase assay*

ATPase activity was measured at 37°C in a reaction medium (final vol. 0.5 ml) containing 40 mM Tris–HCl, 20 μg pyruvate kinase, 2 mM phosphoenolpyruvate, 10 mM ATP, 5 mM $MgCl_2$, final pH 8.0. The reaction was started by the addition of BF_1 or TF_1 (5 μl corresponding to ~5 μg protein) and after 2 min at 37°C, it was stopped by 0.2 ml of ice-cold trichloroacetic acid (50%, w/v). The amount of P_i released was determined by the Fiske and SubbaRow method [4].

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

2.3. *$^{32}P_i$ and [^{32}P]ANPP binding assays*

Binding of $^{32}P_i$ or [^{32}P]ANPP to BF_1 in the dark was performed by the centrifugation–elution method in [5] for binding of $^{32}P_i$ to MF_1. In brief, 20 μg BF_1 in 20 μl were added to 70 μl TMMg or TMES buffer (pH 7.5) and 10 μl $^{32}P_i$ or [^{32}P]ANPP. After a 20 min incubation at 20–25°C, the mixture was passed on a short Sephadex column to separate free and bound $^{32}P_i$. Radioactivity was determined by liquid scintillation.

2.4. *Protein determination*

The protein concentration was assayed by the Bradford dye binding method [6]. Bovine serum albumin was used as a standard.

2.5. *Polyacrylamide gel electrophoresis and fluorography*

SDS slab gel electrophoresis in 10% polyacrylamide supplemented with 0.5% linear polyacrylamide was done by the Weber and Osborn method [7]. Fluorography was carried out by using EN^{3}HANCE as described by the manufacturer.

2.6. *Photoinactivation of BF_1 and TF_1 by [^{32}P]ANPP*

Prior to photoirradiation, BF_1 or TF_1 was preincubated in the dark with [^{32}P]ANPP for 20 min at 20–25°C. Photoirradiation by an 250 W Osram halogen lamp was performed as in [1]. Photolabeled BF_1 and TF_1 were freed of the non-bound photolabel by passage on a short Sephadex column [1,5]. The excluded fraction was assayed for bound radioactivity, protein and ATPase activity.

3. RESULTS

3.1. *Binding of P_i to BF_1: Effect of ANPP in the dark on P_i binding*

We found that 1 mol BF_1 was able to bind 1 mol P_i with a K_d of 10 μM when the binding assay was done in the presence of $MgSO_4$ (TMMg buffer) (not shown). This value is nearly 4-times lower than that for P_i binding to MF_1 [1,5]. There was virtually no significant binding of P_i to BF_1 in TMES buffer (< 0.02 mol/mol BF_1, even when 200 μM $^{32}P_i$ was used), a result similar to that reported for MF_1 [5].

Studies on direct binding of [^{32}P]ANPP to BF_1 carried out in the dark were hampered, due to strong interaction between the Sephadex gel and phenol derivatives [8,9]. To obviate this technical difficulty, it was thought that binding of ANPP to BF_1 in the dark would be assayed indirectly by determination of its competitive efficiency against $^{32}P_i$ binding, using the centrifugation–elution method [5]. It may be recalled that, by this method, ANPP was found to compete efficiently against P_i for binding to MF_1 in the dark [1]. This is not the case for BF_1. The difficulty to demonstrate competition between P_i and ANPP for BF_1 in the dark was not due to the absence of ANPP binding site(s) on BF_1 (in fact as shown hereafter in photoactivation experiments, ANPP binds to BF_1 with a well-defined stoichiometry), but most likely to an unfavorable ratio of affinities of P_i and ANPP for BF_1; indeed P_i binds with a nearly 4-times higher efficiency to BF_1 (K_d 10 μM) than to MF_1 (K_d 37 μM) [1,5].

3.2. *Photoinactivation of BF_1 by ANPP*

Preincubation of BF_1 with ANPP in the dark followed by photoirradiation resulted in loss of enzymatic activity either in TMES or TMMg buffer. In both cases the half-maximal effect was observed at ~60 μM ANPP (fig.1). On the other hand, no photoinactivation of BF_1 was found when the enzyme was photoirradiated with 200 μM ANP (the precursor of ANPP) in either TMES or TMMg buffer.

3.3. *Photoaffinity labeling of BF_1 and TF_1 by [^{32}P]ANPP: Correlation between incorporation of [^{32}P]ANPP and ATPase inactivation and identification of the photolabeled subunit(s)*

Photoinactivation of BF_1 was accompanied by covalent incorporation of $^{32}P_i$ radioactivity into BF_1 as shown by the bound radioactivity after SDS–polyacrylamide gel electrophoresis (see below). The amount of bound [^{32}P]ANPP was linearly related to the loss of ATPase activity, whatever the buffer used, i.e., TMMg or TMES; by extrapolating binding data to zero activity, it was found that complete inactivation of BF_1 required 1 mol [^{32}P]ANPP bound/mol BF_1 (fig.2). To identify which subunit(s) of BF_1 react(s) with ANPP, SDS–polyacrylamide slab gel elec-

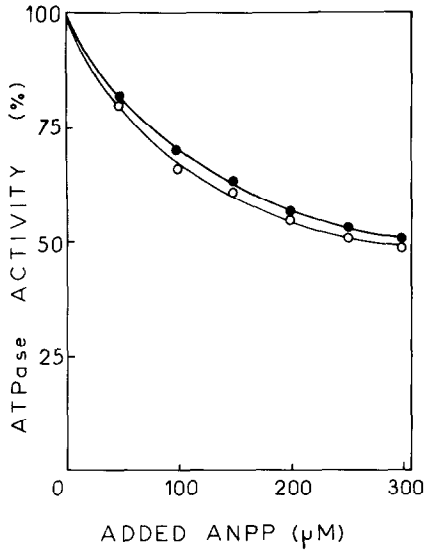

Fig.1. Photoinactivation of BF_1 by increasing ANPP concentrations in TMES buffer and in TMMg buffer. BF_1 was preincubated in the dark for 20 min with various ANPP concentrations and then photoirradiated for 30 min at 22°C. ATPase activity was measured as in section 2: (○) TMES buffer; (●) TMMg buffer.

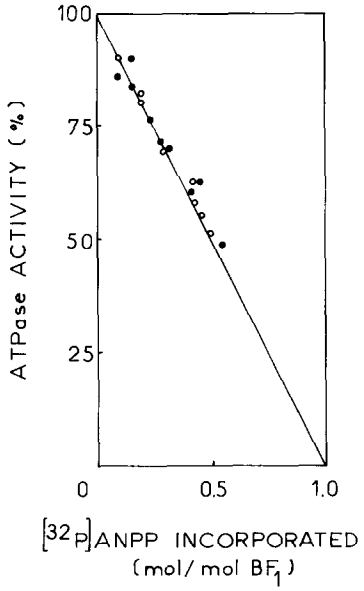

Fig.2. Correlation between photoinactivation of BF_1 and covalent binding of [^{32}P]ANPP. Photoinactivation of BF_1 was done with up to 300 μM [^{32}P]ANPP in TMES buffer (○) and in TMMg buffer (●). After a 20 min preincubation in the dark, followed by a 30 min photoirradiation at 22°C, ATPase activity, bound radioactivity and protein concentration were determined as in section 2.

trophoresis was performed with samples of BF_1 photoinactivated by [^{32}P]ANPP. The α- and β-subunits were characterized by specific modifiers, namely [^{14}C]NEM for the α-subunit and [^{14}C]DCCD for the β subunit [3]. The radioactivity was found to be located only in the β subunit, independent of whether photoirradiation was carried out in TMMg or TMES buffer (fig.3, tracks 3 and 5). Addition of P_i prior to that of ANPP followed by photoirradiation fully protected BF_1 against photolabeling (fig.3, tracks 4 and 6), whatever the buffer used.

Mesophilic BF_1 and MF_1 dissociate, if a stabilizing buffer is not used, in contrast to the thermophilic TF_1 which is much more stable [10]. Consequently it was interesting to photolabel TF_1 with [^{32}P]ANPP. Photoinactivation of TF_1 by [^{32}P]ANPP was more effective than that of BF_1, 85% photoinactivation being obtained with 150 μM ANPP in TMMg medium (fig.4 cf. fig.1).

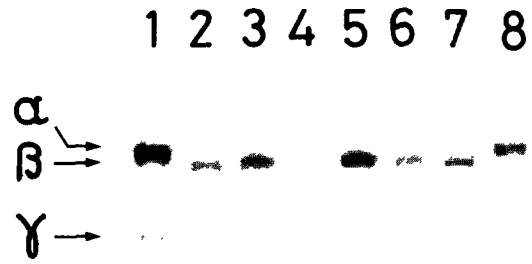

Fig.3. Photolabeling of BF_1 by [^{32}P]ANPP. BF_1 photolabeled as in section 2 was analyzed by SDS–polyacrylamide gel electrophoresis followed by fluorography: (1,8) BF_1 labeled in the α, γ and ϵ subunits with [^{14}C]NEM; (2,7) BF_1 labeled in the β subunit with [^{14}C]DCCD; (4) BF_1 photolabeled with 200 μM [^{32}P]ANPP in the presence of 10 mM P_i in TMES buffer; (3) BF_1 photolabeled with 200 μM [^{32}P]ANPP in TMES buffer; (6) BF_1 photolabeled with 200 μM [^{32}P]ANPP in the presence of 10 mM P_i in TMMg buffer; (5) BF_1 photolabeled with 200 μM [^{32}P]ANPP in TMMg buffer. Identical amounts (4 μg) of photolabeled BF_1 were analyzed.

Yet, the photolabeling stoichiometry for TF_1 was the same as for BF_1; i.e., 1 mol [^{32}P]ANPP incorporated/mol TF_1 for full inactivation of the enzyme (fig.4). The bound [^{32}P]ANPP was localized only on the β-subunit (fig.5). As for BF_1, there was a good protection by P_i against ANPP photoinactivation of TF_1 (not shown), and against photoincorporation of [^{32}P]ANPP into TF_1 (fig.5).

3.4. *Effect of P_i, ADP and ATP on the photolabeling of BF_1 by [^{32}P]ANPP*

P_i added to BF_1 prior to the photoinactivation step protected efficiently BF_1 against photoinactivation by [^{32}P]ANPP. This protection was accompanied by a decreased incorporation of [^{32}P]ANPP (table 1). The photolabeling stoichiometry, when extrapolated to full inactivation, under conditions of partial protection by P_i, remained the same as in the absence of protection by P_i; i.e., 1 mol [^{32}P]ANPP incorporated/mol BF_1.

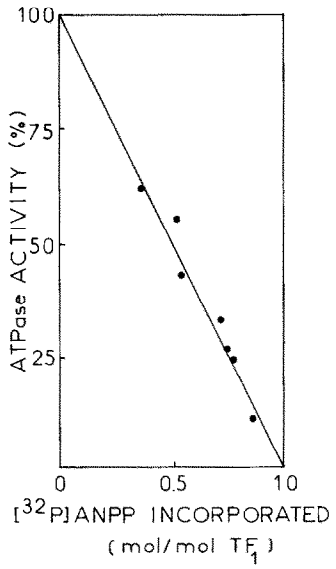

Fig.4. Correlation between photoinactivation of TF_1 and covalent binding of [^{32}P]ANPP. Photoinactivation of TF_1 was done with up to 300 μM [^{32}P]ANPP in TMMg buffer. After a 20 min preincubation in the dark, followed by a 30 min photoirradiation, at 22°C, ATPase activity, bound radioactivity and protein concentrations were determined as in section 2.

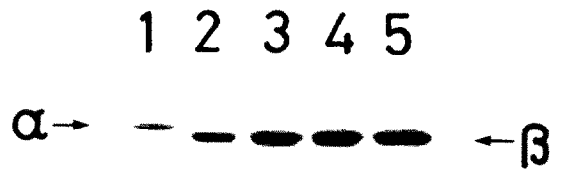

Fig.5. Photolabeling of TF_1 by [^{32}P]ANPP. Analysis of the photolabeled peptides in TF_1 by SDS–polyacrylamide gel electrophoresis followed by fluorography: (1) TF_1 labeled with [^{14}C]NEM in the α subunit; (2) TF_1 photolabeled with 250 μM [^{32}P]ANPP in the presence of 10 mM P_i; (3) TF_1 photolabeled with 150 μM [^{32}P]ANPP; (4) TF_1 photolabeled with 200 μM [^{32}P]ANPP; (5) TF_1 photolabeled with 250 μM [^{32}P]ANPP.

ADP and ATP also partially protected BF_1 against photoinactivation by [^{32}P]ANPP and concomitant photolabeling (table 1). As for P_i protection, the photolabeling stoichiometry under conditions of partial protection by ADP or ATP was the same as in the absence of ADP or ATP, when extrapolated to full inactivation.

3.5. *Is it really the P_i binding site on BF_1 which is photolabeled by ANPP?*

$^{32}P_i$ binding experiments were carried out on BF_1 photoirradiated alone (control experiment) and on BF_1 photolabeled and photoinactivated by ANPP to an extent of ~50%. The amount of $^{32}P_i$ incorporated into BF_1 was reduced from 1 mol/mol BF_1 to 0.5 mol, the K_d value remaining the same (10 μM) (fig.6). The parallelism between the extent of photolabeling and the decrease of P_i binding shows unambiguously that ANPP labels the P_i site of BF_1.

Table 1

Effect of P_i, ADP and ATP on the photoinactivation of BF_1 by [^{32}P]ANPP

Buffer used	[^{32}P]ANPP (μM)	Additions	% Remaining ATPase activity	Bound [^{32}P]ANPP (mol/mol BF_1)
TMMg	50	none	72	0.29
TMMg	50	10 mM P_i	89	0.14
TMMg	150	none	61	0.45
TMMg	150	1 mM P_i	80	0.19
TMMg	150	10 mM P_i	86	0.09
TMES	150	none	57	0.42
TMES	150	1 mM P_i	65	0.30
TMES	200	none	63	0.43
TMES	200	10 mM P_i	82	0.20
TMES	250	none	49	0.55
TMES	250	10 mM P_i	79	0.24
TMMg	150	none	59	0.45
TMMg	150	0.5 mM ADP	80	0.22
TMMg	150	2 mM ADP	84	0.16
TMES	150	none	57	0.42
TMES	150	2 mM ADP	86	0.12
TMES	150	2 mM ATP	90	0.12

BF_1 (1 mg/ml) was preincubated in the dark at 22°C for 20 min in TMMg or TMES buffer, in the presence of P_i, ADP or ATP at the indicated concentration. This was followed by a 30 min photoirradiation. ATPase activity was then measured and the incorporated radioactivity determined (cf. section 2)

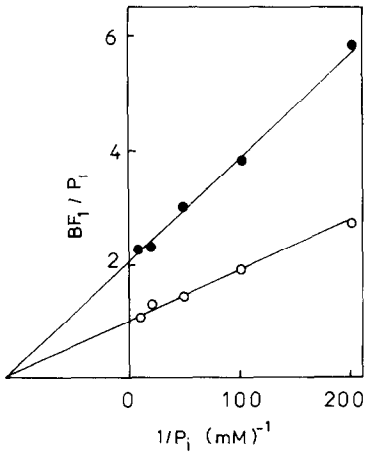

Fig.6. $^{32}P_i$ binding by BF_1 and ANPP-photoinactivated BF_1. $^{32}P_i$ binding experiments were carried out as in section 2 on control BF_1 (○) and on BF_1 inactivated to ~50% by photoirradiation with ANPP(●).

4. DISCUSSION

The following data provide conclusive evidence that photoinactivation of BF_1 by ANPP occurs at the P_i binding site of the enzyme:

(1) ANP, the non-phosphorylated precursor of ANPP has no effect on the ATPase activity upon photoirradiation;

(2) The maximal amount of P_i bound to BF_1 and of [^{32}P]ANPP photoincorporated into BF_1 (when extrapolated to 100% photoinactivation) are the same; i.e., 1 mol/mol BF_1;

(3) BF_1, when photolabeled by ANPP, binds less P_i; the percentage by which P_i binding is reduced is equal to the percentage of photolabeling;

(4) P_i protects BF_1 both against photoinactivation and photolabeling by [^{32}P]ANPP.

One might argue that ADP and ATP also protect MF_1 against photoinactivation by ANPP. It is known however, that ADP and ATP prevent P_i binding to MF_1 [5], which may explain their effect on ANPP binding.

These results allow extension of findings on photolabeling of MF_1 [1] and CF_1 [11], which showed that only the β-subunit of the two enzymes were photolabeled by ANPP and that full inactivation corresponded to the binding of 1 mol ANPP/mol enzyme. There is, as yet, no definite evidence that ANPP labels the catalytic P_i site on BF_1, or on the other F_1-ATPases. However, the specific labeling of the β subunit by ANPP in MF_1, BF_1, TF_1 and CF_1 (here, [1,11]) makes it evident that a recognition site for P_i, either catalytic or regulatory, is located on the β-subunit of these ATPases.

ACKNOWLEDGEMENTS

We are grateful to Professor Y. Kagawa for providing us purified TF_1. This work was supported by a grant from the Fondation pour la Recherche Médicale.

REFERENCES

[1] Lauquin, G., Pougeois, R. and Vignais, P.V. (1980) Biochemistry 19, 4620–4626.
[2] Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7910–7916.
[3] Satre, M., Lunardi, J., Pougeois, R. and Vignais, P.V. (1979) Biochemistry 18, 3134–3140.
[4] Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
[5] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[6] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[7] Weber, K. and Osborn, H. (1969) J. Biol. Chem. 244, 4406–4412.
[8] Brook, A.J.W. and Munday, K.C. (1970) J. Chromatogr. 47, 1–8.
[9] Determan, H. and Walter, I. (1968) Nature 219, 604–605.
[10] Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525–9533.
[11] Pougeois, R., Lauquin, G.J.-M. and Vignais, P.V. (1982) in: Second European Bioenergetics Conference (IUB–IUPAB Bioenergetics Groups) EBEC Reports, vol.2, pp.115–116, LBTM-CNRS Edition, Villeurbanne.