Chantot, J. F. (1972) *Arch. Biochem. Biophys. 153*, 347–356.
Egami, F., Takahashi, K., & Uchida, T. (1964) *Prog. Nucleic Acid Res. Mol. Biol. 3*, 59–101.
Epinatjeff, C., & Pongs, O. (1972) *Eur. J. Biochem. 26*, 434–441.
Heinemann, U., & Saenger, W. (1982) *Nature (London) 299*, 27–31.
Ikehara, M., & Imura, J. (1981) *Chem. Pharm. Bull. 29*, 2408–2412.
Inagaki, F., Kawano, Y., Shimada, I., Takahashi, K., & Miyazawa, T. (1981) *J. Biochem. (Tokyo) 89*, 1185–1195.
Irie, M. (1967) *J. Biochem. (Tokyo) 61*, 550–554.
Irie, M. (1968) *J. Biochem. (Tokyo) 63*, 649–653.
Irie, M. (1970) *J. Biochem. (Tokyo) 68*, 69–79.
Kanaya, S., & Uchida, T. (1981) *J. Biochem. (Tokyo) 89*, 591–597.
Kimura, S., Matsuo, H., & Narita, K. (1979) *J. Biochem. (Tokyo) 85*, 301–310.
Kyogoku, Y., Watanabe, M., Kainosho, M., & Oshima, T. (1982) *J. Biochem. (Tokyo) 91*, 675–679.
Matsuda, A., & Ueda, T. (1981) *Nippon Kagaku Kaishi 5*, 845–850.
Oshima, T., & Imahori, K. (1971a) *J. Biochem. (Tokyo) 69*, 987–990.
Oshima, T., & Imahori, K. (1971b) *J. Biochem. (Tokyo) 70*, 197–199.
Pongs, O. (1970) *Biochemistry 9*, 2316–2321.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647–806.
Richarz, R., & Wüthrich, K. (1978) *J. Magn. Reson. 30*, 147–150.
Rüterjan, H., & Pongs, O. (1971) *Eur. J. Biochem. 18*, 313–318.
Sato, K., & Egami, F. (1957) *J. Biochem. (Tokyo) 44*, 753–767.
Sawada, F., & Irie, M. (1969) *J. Biochem. (Tokyo) 66*, 415–418.
Son, T. D., Guschlbauer, W., & Guêron, M. (1972) *J. Am. Chem. Soc. 94*, 7903–7911.
Takahashi, K. (1961) *J. Biochem. (Tokyo) 60*, 239–245.
Takahashi, K. (1965) *J. Biol. Chem. 240*, 4117–4119.
Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
Takahashi, K. (1970a) *J. Biochem. (Tokyo) 67*, 833–839.
Takahashi, K. (1970b) *J. Biochem. (Tokyo) 68*, 659–664.
Takahashi, K. (1971) *J. Biochem. (Tokyo) 69*, 331–338.
Takahashi, K. (1972) *J. Biochem. (Tokyo) 72*, 1469–1481.
Takahashi, K. (1974) *Yuki Gosei Kagaku Kyokaishi 32*, 298–316.
Takahashi, K., Stein, W. H., & Moore, S. (1967) *J. Biol. Chem. 242*, 4682–4690.
Walz, F. G. (1976) *Biochemistry 15*, 4446–4450.
Walz, F. G. (1977) *Biochemistry 16*, 4568–4571.
Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175*, 558–564.

Further Investigations on the Inorganic Phosphate Binding Site of Beef Heart Mitochondrial F_1-ATPase†

Richard Pougeois* and Guy J.-M. Lauquin‡

Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 38041 Grenoble Cedex, France

Received March 14, 1984; Revised Manuscript Received August 8, 1984

ABSTRACT: The possibility that 4-azido-2-nitrophenyl phosphate (ANPP), a photoreactive derivative of inorganic phosphate (P_i) [Lauquin, G., Pougeois, R., & Vignais, P. V. (1980) *Biochemistry 19*, 4620–4626], could mimic ATP was investigated. ANPP was hydrolyzed in the dark by sarcoplasmic reticulum Ca^{2+}-ATPase in the presence of Ca^{2+} but not in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. ANPP was not hydrolyzed by purified mitochondrial F_1-ATPase; however, ADP and ATP protected F_1-ATPase against ANPP photoinactivation. On the other hand, the trinitrophenyl nucleotide analogues (TNP-ADP, TNP-ATP, and TNP-AMP-PNP), which bind specifically at the two catalytic sites of F_1-ATPase [Grubmeyer, C., & Penefsky, H. (1981) *J. Biol. Chem. 256*, 3718–3727], abolished P_i binding on F_1-ATPase; they do not protect F_1-ATPase against ANPP photoinactivation. Furthermore, ANPP-photoinactivated F_1-ATPase binds the TNP analogues in the same way as the native enzyme. The P_i binding site of F_1-ATPase, which is shown to be photolabeled by ANPP, does not appear to be at the γ-phosphate position of the catalytic sites.

Purified beef heart mitochondrial F_1-ATPase is a cold-labile enzyme that contains five distinct subunits [for a review, see Penefsky (1979) and Senior (1979)]. F_1-ATPase[1] exhibits a single binding site for P_i (Penefsky, 1977; Kasahara & Penefsky, 1978). In an attempt to photolabel the P_i binding site of F_1-ATPase, ANPP, an azido derivative of P_i, was prepared; its binding properties were described in a preceding paper (Lauquin et al., 1980). The criteria for the binding of ANPP

† These investigations were supported in part with a grant from the Fondation pour la Recherche Médicale.

* Address correspondence to this author at the Laboratoire d'Hormonologie, Centre Hospitalier Régional et Universitaire, BP 217X, 38043 Grenoble Cedex, France.

‡ Present address: Laboratoire de Physiologie Cellulaire, 13288 Marseille Cedex 9, France.

[1] Abbreviations: F_1-ATPase, mitochondrial F_1-ATPase; P_i, inorganic phosphate; ANPP, 4-azido-2-nitrophenyl phosphate; ANP, 4-azido-2-nitrophenol; AMP-PNP, adenyl-5′-yl imidodiphosphate; TNP-ADP, TNP-ATP, and TNP-AMP-PNP, the 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene) derivatives of ADP, ATP, and AMP-PNP (at neutral or basic pH); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

0006-2960/85/0424-1020$01.50/0

to the P_i binding site of F_1-ATPase were as the follows: (1) in the dark ANPP behaved as a competitive inhibitor of P_i binding; (2) P_i added prior to photoirradiation protected the enzyme against photoinactivation and it prevented the covalent binding of [^{32}P]ANPP; (3) ANP, the precursor of ANPP, had no effect on P_i or ADP binding; (4) no photoinactivation was found when ANP was used instead of ANPP.

On the other hand, on the basis of the following findings, our attention was directed to the fact that ANPP could also behave as an ADP or ATP analogue. (1) ANPP was hydrolyzed to give P_i and ANP by sarcoplasmic reticulum Ca^{2+}-ATPase; (2) ADP or ATP protected F_1-ATPase against photoinactivation by ANPP. A number of complementary experiments dealing with ANPP and F_1-ATPase were therefore carried out. They are described in the present paper. For the sake of clarity, the results are presented in three parts. Part 1 consists of experiments in which ANPP was found to mimic ATP; part 2 shows that ANPP is indeed a P_i analogue for F_1-ATPase. Finally, in part 3, we consider the possibility that the phosphate residue of ANPP might be equivalent to the γ-phosphate moiety of ATP at the catalytic sites of the enzyme.

Experimental Procedures

Materials

ADP, ATP, AMP-PNP, phosphoenolpyruvate, 3-phosphoglyceric acid, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase were obtained from Boehringer Mannheim. Charcoal was purchased from Sigma and picrylsulfonic acid from Aldrich. Carrier-free [^{32}P]P_i was purchased from the Commissariat à l'Energie Atomique (Saclay, France). All other chemicals were of reagent-grade quality. ANP and ANPP were synthesized and purified as previously described (Lauquin et al., 1980). TNP-ATP was synthesized and purified as described by Hiratsuka & Uchida (1973). TNP-ADP was obtained via TNP-ATP hydrolysis by F_1-ATPase, and TNP-[γ-^{32}P]ATP was synthesized according to Grubmeyer & Penefsky (1981a). TNP-AMP-PNP was synthesized and purified as described for TNP-ATP (Hiratsuka & Uchida, 1973). However, because of the presence of contaminants, TNP-AMP-PNP was further purified by descending chromatography on Whatman No. 3 paper in 1-butanol/acetic acid/H_2O (5/2/3 v/v/v). The purity of the TNP derivatives was monitored by analytical thin-layer chromatography on polyethylenimine–cellulose plates developed with 2 M formic acid and 0.5 M LiCl. Their concentrations were determined at pH 8.0 from the molar absorption coefficient ϵ_{408} = 26 400 M^{-1} cm^{-1}. When examined at 470 nm, they had an ϵ_{470} equal to 18 500 M^{-1} cm^{-1}, in agreement with Hiratsuka & Uchida (1973).

Methods

Preparation of F_1-ATPase. F_1-ATPase was prepared and stored as an ammonium sulfate suspension (Knowles & Penefsky, 1972). Before use, an aliquot was centrifuged, the pellet was dissolved in 0.25 M sucrose/50 mM Tris–acetate, pH 7.5, and desalted by centrifugation through Sephadex (G-50, fine) columns equilibrated in the same buffer (Penefsky, 1977). The concentration of F_1-ATPase was calculated by using a molecular mass of 360 000 (Lambeth et al., 1971). The protein concentration was determined by the dye-binding method using Coomassie blue G-250 as described by Bradford (1976). Bovine serum albumin was used as a standard.

Preparation of Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles were prepared from rabbit muscles as described by Dupont et al. (1982).

Table I: Effect of ADP and ATP on Photoinactivation of F_1-ATPase by ANPP[a]

additions	F_1-ATPase remaining act. (% of control)	additions	F_1-ATPase remaining act. (% of control)
none	48	0.2 mM ATP	53
0.2 mM ADP	57	0.5 mM ATP	63
0.5 mM ADP	67	1 mM ATP	77
1 mM ADP	65	5 mM ATP	79

[a] F_1-ATPase (0.8 mg/mL) was preincubated in the dark at 20 °C in EDTA buffer (cf. Methods) containing 150 μM ANPP in the presence of ADP and ATP as indicated. After 25 min, the samples were photoirradiated for 30 min at 20 °C and then the ATPase activity was determined as described under Methods.

Enzymatic Assays. Measurement of F_1-ATPase activity was carried out at 30 °C. The reaction medium contained 40 mM Tris-HCl, 10 mM ATP, 5 mM $MgCl_2$, 20 μg of pyruvate kinase, and 2 mM phosphoenolpyruvate, final pH 8.0, final volume 0.5 mL. The reaction was started by addition of an aliquot fraction of the F_1-ATPase (5 μL) and stopped after 2 min by addition of 0.2 mL of ice-cold trichloroacetic acid, 50% (w/v). The released P_i was determined by the Fiske & SubbaRow (1925) method. Measurement of TNP-ATPase activity was carried out at room temperature. The reaction mixture contained 40 mM Tris-HCl, 10 μM TNP-[γ-^{32}P]-ATP, and 10 mM $MgCl_2$, final volume 1 mL, final pH 8.0. The reaction was started by addition of 10 μg of F_1-ATPase and stopped after 30 s by addition of 0.4 mL of ice-cold trichloroacetic acid, 50% (w/v). The released [^{32}P]P_i was determined as described by Grubmeyer & Penefsky (1981a). Incubation of [^{32}P]ANPP with sarcoplasmic reticulum ATPase was carried out at 20 °C in the dark. The medium contained 50 mM Mops, 50 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 100 μM [^{32}P]ANPP, and vesicles (0.9 mg/mL). After 10 min, the suspension was centrifuged and an aliquot of the supernatant was analyzed by ascending chromatography on Whatman No. 1 paper, in diisopropyl ether/98% formic acid/H_2O (90/60/3 v/v/v), followed by autoradiography.

Photoinactivation of F_1-ATPase by [^{32}P]ANPP. The reaction mixture, in stoppered glass tubes, contained 80 μL of [^{32}P]ANPP, 50 mM Tris, 50 mM MES and 1 mM $MgSO_4$ (or 1 mM EDTA when ATP was present; Table I), final pH 7.5; the reaction was initiated by 20 μL of F_1-ATPase (about 100 μg), desalted as described above. After 25-min preincubation in the dark at room temperature, the samples were photoirradiated for 30 min. The light source was a 250-W Osram halogen lamp. The tubes, partially immersed in a water bath maintained at 20–25 °C, were rotated nearly horizontally. A glass plate was placed between the light source and the glass tubes to eliminate ultraviolet light, which is deleterious for the enzyme (data not shown). Under the above conditions F_1-ATPase photoirradiated alone (without ANPP) remained fully active. After photoirradiation the ATPase activity was measured; the samples were either left for 20 min or incubated with 10 mM P_i for 20 min (cf. Results); then they were applied to the top of a centrifuge column equilibrated with the same buffer, and after centrifugation, the protein content and the radioactivity were determined.

TNP Derivative Binding Measurements. Binding of TNP derivatives was measured at room temperature by absorbance difference spectra taken in a Perkin-Elmer double-beam spectrophotometer with 1-mL samples in cuvettes of 1-cm light path. The reference cell contained 0.25 M sucrose, 50 mM Tris-HCl, 1 mM KP_i, and 10 mM $MgSO_4$, pH 7.5, and the sample cell F_1-ATPase in the same buffer. Reagents were added with Hamilton syringes and allowed to be in contact

for 5 min for stabilization. Then the spectrum was taken between 350 and 500 nm. The difference in absorbance between the peak at 425 nm and the trough at 390 nm was used [cf. Grubmeyer & Penefsky (1981a)].

RESULTS

Is ANPP an ATP Analogue? The following data indicate that ANPP is a substrate for the sarcoplasmic reticulum Ca^{2+}-ATPase in contrast to F_1-ATPase (Lauquin et al., 1980). ANPP was hydrolyzed by the sarcoplasmic reticulum Ca^{2+}-ATPase in the hydrolysis standard conditions, i.e., in the presence of Ca^{2+} (cf. Methods). A control experiment performed without Ca^{2+} (i.e., in the presence of 1 mM EGTA) did not reveal significant hydrolysis of ANPP. However, when the Ca^{2+}-ATPase was photoirradiated with [^{32}P]ANPP in the presence of EGTA, some covalently bound radioactivity was recovered (data not shown). From these results, it appears that ANPP could mimic ATP to the Ca^{2+}-ATPase in the dark and even in EGTA medium when photoirradiated. The fact that F_1-ATPase or sarcoplasmic reticulum Ca^{2+}-ATPase in EGTA medium did not hydrolyze ANPP in the dark does not imply that ANPP is not an ATP analogue, and more generally, the fact that a product might be a substrate analogue for an enzyme does not imply its hydrolysis. For example, TNP-ATP, which is specifically recognized by F_1-ATPase (Grubmeyer & Penefsky, 1981a), sarcoplasmic reticulum Ca^{2+}-ATPase (Dupont et al., 1982), and the Na^+/K^+-ATPase (Moczydlowski & Fortes, 1981), is hydrolyzed only by the two first enzymes and not by the third one.

Protective Effect of ADP and ATP on the ANPP Photoinactivation of F_1-ATPase. As can be seen from Table I, ADP protected partially F_1-ATPase against photoinactivation by ANPP, the maximal effect being obtained with about 0.5 mM ADP. When ATP was used, a better protection was obtained, and the maximal effect was at about 1 mM ATP (Table I). Although these results might mean that ANPP binds at the nucleotide site(s) of F_1-ATPase, it should be remembered that ADP and ATP prevent P_i binding by F_1-ATPase (Penefsky, 1977) and that they partially protect purified bacterial and chloroplast ATPases against photoinactivation by ANPP (Pougeois et al., 1983a,b).

We have previously shown that, in the dark, there was a competitive inhibition of P_i binding by ANPP (Lauquin et al., 1980); this result is consistent with the proposal that ANPP is a phosphate analogue; it does disprove the alternative that ANPP is an adenine nucleotide analogue since ADP (or ATP) prevents P_i binding by F_1-ATPase (Penefsky, 1977).

Direct Demonstration That ANPP Is a True P_i Analogue for F_1-ATPase. In a previous paper concerning the interaction of ANPP with F_1-ATPase (Lauquin et al., 1980), the "caged P_i" property of ANPP was shown (the term "caged" was introduced by Kaplan et al. (1978) to describe the instability in light of the phosphate bound in 2-nitrobenzyl phosphate). For this reason, when photolabeling F_1-ATPase with [^{32}P]-ANPP was performed, we had to incubate the reaction mixture, after photoirradiation, with 10 mM cold P_i for 20 min to dilute the [^{32}P]P_i released by photohydrolysis of [^{32}P]ANPP; this enabled us to measure the amount of [^{32}P]P_i covalently bound to the enzyme; it should be remembered that photohydrolysis of [^{32}P]ANPP was about 50%, after 30-min photoirradiation; i.e., there was 50% free [^{32}P]P_i in the reaction mixture [cf. Lauquin et al. (1980)].

Advantage has been taken of the caged compound to demonstrate that we really photolabel the P_i binding site on F_1-ATPase. Schematically, the principle of this experiment was as follows. After photoirradiation with 200 μM [^{32}P]ANPP, the reaction mixture contained about 100 μM [^{32}P]P_i, which is a nearly saturating concentration for the P_i binding site of F_1-ATPase (Penefsky, 1977; Lauquin et al., 1980), and 0.5 mol of covalent bound [^{32}P]ANPP/mol of F_1-ATPase. Thus, two situations may occur: (1) if ANPP is indeed located at the P_i binding site, after centrifugtion–elution of the mixture (cf. Methods), we should find 1 mol of [^{32}P]P_i/mol of F_1-ATPase, as in the case of incubation of F_1-ATPase with 100 μM [^{32}P]P_i; (2) if ANPP labels a different site, then we should find 1.5 mol of [^{32}P]P_i bound/mol of F_1-ATPase, i.e., 0.5 mol of [^{32}P]ANPP, covalently bound, plus 1 mol of [^{32}P]P_i corresponding to the specific P_i binding site described by Penefsky (1977). As shown in Table II, there was no more than 1 mol of [^{32}P]P_i bound/mol of F_1-ATPase. This is the direct proof that ANPP is a true P_i analogue for F_1-ATPase. Therefore, ANPP appears to be an appropriate probe to investigate the role of the P_i binding site on F_1-ATPase.

Table II: ANPP Is a True P_i Analogue for F_1-ATPase[a]

ANPP added (μM)	photoinactivation (%)	^{32}P covalently bound (mol/mol of F_1)	^{32}P bound (mol/mol of F_1)
100	47	0.44	0.84
200	57	0.59	0.94
250	58	0.60	0.89
300	62	0.66	1.02

[a] F_1-ATPase was photoinactivated by [^{32}P]ANPP at increasing concentrations as described under Methods. After photoirradiation, the extent of inactivation, the amount of ^{32}P covalently bound, and the total ^{32}P bound were determined as detailed under Methods.

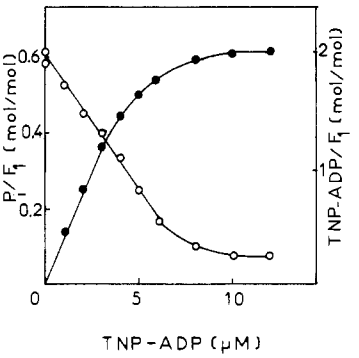

FIGURE 1: Binding of P_i and TNP-ADP to F_1-ATPase. (○) Binding of P_i. F_1-ATPase (1 mg/mL) was preincubated for 20 min with 60 μM [^{32}P]P_i as described under Methods. Then 90 μL of F_1-ATPase was added to a series of tubes containing 10 μL of buffer supplemented with TNP-ADP at the final concentrations shown in the abscissa. After 10 min, the samples were applied to a series of centrifuge columns and the bound radioactivity was measured (cf. Methods). TNP-ADP binding (●) was measured as described under Methods.

Are the Catalytic Sites on F_1-ATPase Affected by ANPP? In this part of the work, we used the TNP derivatives of ADP, ATP, and AMP-PNP in order to study their possible interactions with the P_i or ANPP binding site on F_1-ATPase. Grubmeyer & Penefsky (1981a) recently showed that F_1-ATPase contains two hydrolytic sites for TNP-ATP; these two high-affinity binding sites are also probed by TNP-ADP.

As shown in Figure 1, upon binding of 1 mol of TNP-ADP/mol of F_1-ATPase, P_i binding was inhibited to about 50%; for 2 mol of TNP-ADP bound to 1 mol of F_1-ATPase, P_i binding was nearly abolished. Similar results were obtained when using TNP-ATP instead of TNP-ADP (data not shown). However, since these experiments were carried out in Mg^{2+} medium [P_i binds to F_1-ATPase only in the presence of Mg^{2+}; cf. Penefsky (1977)], TNP-ATP was at least partially hydrolyzed during the course of the incubation (Grubmeyer & Penefsky, 1981a). Thus, the fact that TNP-ATP abolished

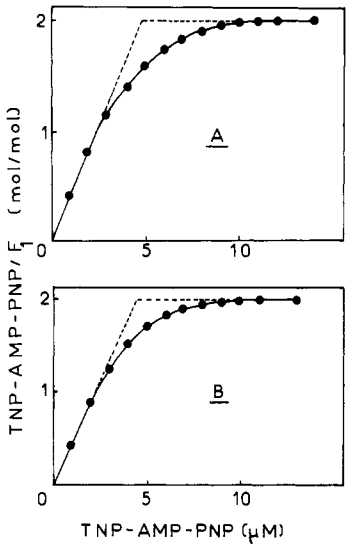

FIGURE 2: TNP-AMP-PNP binding to native F_1-ATPase and ANPP-photoinactivated F_1-ATPase. TNP-AMP-PNP binding was measured as described under Methods with native F_1-ATPase photoirradiated alone without ANPP (A) and with F_1-ATPase photoinactivated by ANPP to an extent of 50% (B). The same results were obtained when 1 mM P_i was omitted from the reaction mixture.

Table III: Photoinactivation of F_1-ATPase by ANPP in the Presence of TNP-ADP, TNP-ATP, and TNP-AMP-PNP[a]

additions	F_1-ATPase remaining act. (% of control)
none	48
20 μM TNP-ADP	50
20 μM TNP-ATP	50
20 μM TNP-AMP-PNP	45

[a] F_1-ATPase (0.9 mg/mL, i.e., 2.5 μM) was preincubated with 150 μM ANPP, in the presence or absence of TNP nucleotide analogues, for 25 min, at room temperature in the dark. After 30-min photoirradiation, TNP-ATPase activity was measured (cf. Methods). F_1-ATPase preincubated with 150 μM ANPP in the absence of TNP analogues was 50% photoinactivated when the ATPase activity was determined with ATP as a substrate.

the P_i binding may mean that either TNP-ATP mimics TNP-ADP or TNP-ATP hydrolysis promotes the release of previously bound P_i. Consequently, we synthesized the TNP derivative of AMP-PNP, a nonhydrolyzable analogue of ATP (Yount et al., 1971; Philo & Selwyn, 1974; Penefsky, 1974). As expected, binding of TNP-AMP-PNP to F_1-ATPase (Figure 2) was identical with the binding of TNP-ADP or TNP-ATP; furthermore, P_i binding was prevented by TNP-AMP-PNP (not shown) as it was by TNP-ADP or TNP-ATP.

We next performed ANPP photoinactivation experiments in the presence of TNP-ADP, TNP-ATP, and TNP-AMP-PNP. As shown in Table III, occupation of the two catalytic sites of F_1-ATPase by theTNP nucleotide analogues did not protect against photoinactivation by ANPP. The ATPase activities shown in Table III were measured with [γ-^{32}P]-TNP-ATP as substrate since the TNP analogues are strong inhibitors of F_1-ATPase activity when the enzymatic activity is tested with ATP as the substrate (Grubmeyer & Penefsky, 1981a). It is noteworthy that in the same experiment, photoinactivation of the sample photoirradiated with ANPP but without TNP analogues (corresponding to line 1 of Table III) was 50% when tested with ATP as the substrate.

TNP-ADP binding assays performed on the ANPP-photoinactivated F_1-ATPase and on the native enzyme did not show any difference in TNP-ADP binding (data not shown). The experiment, however, did not answer the question whether or not the ANPP binding site is located at the γ-phosphate position of the catalytic sites. For this purpose TNP-AMP-PNP binding assays were carried out on the native F_1-ATPase (photoirradiated control) and on the ANPP-photoinactivated F_1-ATPase (50% photoinactivated). In both cases, 2 mol of TNP-AMP-PNP was found to bind per mol of F_1-ATPase (Figure 2). Should the conformation of the catalytic site probed by TNP-AMP-PNP be modified by ANPP at the γ-phosphate position, 1.5 mol of TNP-AMP-PNP only would have bound to 1 mol of F_1-ATPase instead of 2 mol since 0.5 mol of ANPP covalently binds per mol of F_1-ATPase (see above).

These data taken together strongly suggest that ANPP and therefore P_i do not interfere with the TNP nucleotides that are recognized by the catalytic sites of F_1-ATPase.

Discussion

Data from this paper and from earlier studies (Lauquin et al., 1980) clearly demonstrate that ANPP reacts with a P_i binding site on F_1-ATPase. This is based on the following lines of evidence: (1) extrapolation to zero activity corresponds to the covalent binding of 1 mol of ANPP to 1 mol of F_1-ATPase (Lauquin et al., 1980) in accordance with the presence of a single reversible P_i binding site (Penefsky, 1977); (2) ANPP-modified F_1-ATPase binds at maximum 1 mol of P_i, the bound species being either ANPP (covalent binding) or P_i released from ANPP (noncovalent binding) (Table II). Thus, ANPP mimics P_i for binding to F_1-ATPase and the P_i site is located on the β-subunit (Lauquin et al., 1980).

What is the role of this P_i binding site? Penefsky (1977) had suggested that it may well bind to a site equivalent to the γ-phosphate position of ATP. This hypothesis has, however, to be accepted with reservation for the following reasons. (1) If P_i did bind to the catalytic site, then it would be recognized immediately by the enzyme as a substrate; in contrast, the binding plateau is attained in about 15 min (Penefsky, 1977). Furthermore, concerning the interaction of ANPP with F_1-ATPase, in the absence of the 20-min preincubation step in the dark, the above correlation between photoinactivation and incorporation of ANPP did not hold (data not shown). (2) Whereas there are at least two catalytic sites on F_1-ATPase (Grubmeyer & Penefsky, 1981a) or possibly three (Cross & Nalin, 1982; Gresser et al., 1982; Grubmeyer et al., 1982), it is strange that only one high-affinity P_i binding site (Penefsky, 1977) or one ANPP binding site (Lauquin et al., 1980) per F_1-ATPase can be demonstrated. It must, however, be added that a second nonsaturable P_i binding site has been reported (Kasahara & Penefsky, 1978). (3) P_i, as a product of ATP hydrolysis, does not inhibit the enzymatic activity of F_1-ATPase. (4) ATP and ADP prevent equally well P_i binding to the enzyme (Penefsky, 1977).

In this paper, it is shown that P_i binding is nearly abolished by TNP analogues, whether the analogue is hydrolyzed (for example, TNP-ATP) or not hydrolyzed (for example, TNP-ADP and TNP-AMP-PNP). It should be noted that two catalytic sites in F_1-ATPase must be occupied in order to virtually completely abolish P_i binding, the occupancy of one catalytic site resulting in half-inhibition (Figure 1). Strong inhibition of P_i binding by the TNP analogues may be explained on the basis of a large difference in affinity between P_i (K_d = 37 μM; Penefsky, 1977) and TNP nucleotides (K_d too low to be measured for the first site and K_d = 20 nM for the second site) (Grubmeyer & Penefsky, 1981a). The TNP nucleotides would induce a conformational change in the enzyme that causes the release of the previously bound P_i. This interpretation is corroborated by the absence of protection by

TNP-ADP, TNP-ATP, or TNP-AMP-PNP against ANPP photoinactivation (Table III). Furthermore, direct TNP-AMP-PNP (or TNP-ADP) binding assays showed that the two catalytic sites of F_1-ATPase are unmodified in ANPP-photoinactivated F_1-ATPase (Figure 2).

Grubmeyer & Penefsky (1981a,b) have clearly demonstrated that F_1-ATPase contains two cooperative catalytic sites. Although a third binding site on F_1-ATPase for TNP analogues can be filled, they have found that during the steady-state period of TNP-ATP hydrolysis, only two binding sites were occupied by the TNP analogues. It is noteworthy that the third binding site can be filled only after prolonged (30–60 min) incubation with high concentrations of TNP analogues and only in the presence of Mg^{2+}, which reminds the binding properties of P_i. Consequently, this third binding site does not seem to function as a catalytic site. In the presence of EDTA the third site is not filled by TNP-ATP (Grubmeyer & Penefsky, 1981a), and yet F_1-ATPase does hydrolyze TNP-ATP (Pougeois, 1983). We propose that this site is a regulatory one for F_1-ATPase and that it could be the same binding site for P_i, since P_i binding occurs only in the presence of Mg^{2+} and after similar prolonged incubation (Penefsky, 1977). Unfortunately, this hypothesis cannot be experimentally tested with ANPP-modified F_1-ATPase; such an experiment would theoretically distinguish between 100% (i.e., 3 mol of TNP analogue/mol of F_1-ATPase) and 83% (i.e., 2.5 mol of TNP analogue/mol of ANPP-modified F_1-ATPase) for maximum photoinactivation by ANPP. In practice this is more complicated. Two methods may be used: (1) radioactive TNP analogue binding measured by centrifugation–elution and (2) TNP analogue binding measured by the difference spectrum. In the first case, inaccuracy arises from the strong interaction that occurs between phenol derivatives and Sephadex gel (Determan & Walter, 1968; Lauquin et al., 1980; Grubmeyer & Penefsky, 1981a). In the second case, the method is accurate only when the affinity is very high; otherwise, a slight difference in the TNP analogue addition into the two cuvettes (cf. Methods) is sufficient to give rise to an artifactual absorbance difference.

In conclusion, we suggest that the putative regulatory site proposed above in isolated F_1-ATPase might be directly involved in ATP synthesis in the membrane-bound enzyme.

ACKNOWLEDGMENTS

We thank Y. Dupont for providing us with sarcoplasmic reticulum vesicles. We are also grateful to P. V. Vignais for advice and support. Thanks are due to J. Bournet for typing the manuscript.

Registry No. ATPase, 9000-83-3; ANPP, 74784-75-1; ATP, 56-65-5; ADP, 58-64-0; P_i, 14265-44-2; TNP-ATP, 84412-19-1; TNP-ADP, 84412-17-9; TNP-AMP-PNP, 94347-74-7.

REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Cross, R. L., & Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874–2881.
Determan, H., & Walter, I. (1968) *Nature (London) 219*, 604–605.
Dupont, Y., Chapron, Y., & Pougeois, R. (1982) *Biochem. Biophys. Res. Commun. 106*, 1272–1279.
Fiske, C. H., & SubbaRow, Y. (1925) *J. Biol. Chem. 66*, 375–400.
Gresser, M. J., Myers, J. A., & Boyer, P. D. (1982) *J. Biol. Chem. 257*, 12030–12038.
Grubmeyer, C., & Penefsky, H. S. (1981a) *J. Biol. Chem. 256*, 3718–3727.
Grubmeyer, C., & Penefsky, H. S. (1981b) *J. Biol. Chem. 256*, 3728–3734.
Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.
Hiratsuka, T., & Uchida, K. (1973) *Biochim. Biophys. Acta 320*, 635–647.
Kaplan, J. H., Forbush, B., III, & Hoffman, J. F. (1978) *Biochemistry 17*, 1929–1935.
Kasahara, M., & Penefsky, H. S. (1978) *J. Biol. Chem. 253*, 4180–4187.
Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6617–6623.
Lambeth, D. O., Lardy, H. A., Senior, A. E., & Brooks, J. C. (1971) *FEBS Lett. 17*, 330–332.
Lauquin, G., Pougeois, R., & Vignais, P. V. (1980) *Biochemistry 19*, 4620–4626.
Moczydlowski, E. G., & Fortes, P. A. (1981) *J. Biol. Chem. 256*, 2346–2356.
Penefsky, H. S. (1974) *J. Biol. Chem. 249*, 3579–3585.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Penefsky, H. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 223–280.
Philo, R. D., & Selwyn, M. J. (1974) *Biochem. J. 143*, 745–749.
Pougeois, R. (1983) *FEBS Lett. 154*, 47–50.
Pougeois, R., Lauquin, G. J.-M., & Vignais, P. V. (1983a) *FEBS Lett. 153*, 65–70.
Pougeois, R., Lauquin, G. J.-M., & Vignais, P. V. (1983b) *Biochemistry 22*, 1241–1245.
Senior, A. E. (1979) in *Membrane Proteins in Energy Transduction* (Capaldi, R. A., Ed.) pp 233–278, Marcel Dekker, New York and Basel.
Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971) *Biochemistry 10*, 2484–2489.