4-Azido-2-nitrophenyl Phosphate, a New Photoaffinity Derivative of Inorganic Phosphate. Study of Its Interaction with the Inorganic Phosphate Binding Site of Beef Heart Mitochondrial Adenosine Triphosphatase†

Guy Lauquin, Richard Pougeois, and Pierre V. Vignais*

ABSTRACT: 4-Azido-2-nitrophenyl phosphate (ANPP) was synthesized and characterized. ANPP, unlabeled or labeled by ^{32}P, was used as a photoreactive analogue of P_i to study the P_i binding site(s) in isolated F_1-ATPase and inside-out particles from beef heart mitochondria. In the dark, the phosphate bond of ANPP was cleaved by alkaline phosphatase but not by mitochondrial F_1-ATPase. ANPP bound reversibly to the phosphate site of F_1-ATPase, as shown by competitive inhibition of binding of P_i to F_1-ATPase by ANPP in the dark; the K_i value was 60 μM. Upon photoirradiation with visible light, [^{32}P]ANPP bound covalently to F_1-ATPase and inactivated the enzyme. Part of the added ANPP was, however, photolyzed with release of P_i. By extrapolation, it could be calculated that complete inactivation of F_1-ATPase was accompanied by incorporation of ^{32}P radioactivity corresponding to 1 mol of [^{32}P]ANPP per mol of F_1-ATPase. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{32}P]-ANPP-labeled F_1-ATPase revealed only one radioactive peptide with a M_r of 50000. This peptide was characterized as the β subunit of F_1-ATPase by specific labeling with [^{14}C]dicyclohexylcarbodiimide [Pougeois, R., Satre, M., & Vignais, P. V. (1979) *Biochemistry 18*, 1408–1413]. Photoirradiation of inside-out submitochondrial particles with [^{32}P]ANPP resulted in the labeling of two peptides with a M_r of 50000 and 30000–32000; both labelings were significantly decreased by incubation of the particles with P_i prior to photoirradiation. The M_r 50000 peptide is most probably the β subunit of F_1-ATPase; the other peptide might be the P_i carrier protein.

The specific ligand binding site(s) of macromolecules can be identified by photoaffinity labeling (Bayley & Knowles, 1977). We have synthesized a photoreactive derivative of inorganic phosphate (P_i),[1] 4-azido-2-nitrophenyl phosphate (ANPP), and we present here a study of its photochemical properties and of its interaction with F_1-ATPase isolated from beef heart mitochondria. Purified F_1-ATPase is a soluble cold-labile enzyme that contains five distinct subunits [for review, see Senior (1979), Penefsky (1979), Pedersen (1975), and Kozlov & Skulachev (1977)] with at least five binding sites for adenine nucleotides and a single reversible binding site for P_i (Penefsky, 1977; Kasahara & Penefsky, 1977, 1978). The location and the number of the nucleotide binding sites have been investigated by affinity labeling with 5-[*p*-(fluorosulfonyl)benzoyl]adenosine (Esch & Allison, 1978, 1979; Di Pietro et al., 1979) and by photoaffinity labeling with 8-azido-ATP (Wagenvoord et al., 1977) and azidonitrophenyl derivatives of ATP (Russell et al., 1976; Cosson & Guillory, 1979) or ADP (Lunardi et al., 1977). The location of the P_i binding site has not yet been investigated, and this report is the first attempt of photolabeling the P_i site of isolated F_1-ATPase. We also present data on photolabeling of submitochondrial particles by ANPP.

Experimental Section

Materials

Carrier-free $^{32}P_i$ was purchased from the "Commissariat à l'Energie Atomique" (Saclay, France); dilutions of the isotope were stored in plastic containers. [^{32}P]$POCl_3$ was obtained from Amersham, [^{14}C]DCCD (54.5 mCi/mmol) from the "Commissariat à l'Energie Atomique", and [^{14}C]NEM (40 mCi/mmol) from New England Nuclear. ATP, phosphoenolpyruvate, alkaline phosphatase (calf intestine), and pyruvate kinase (in 50% glycerol) were obtained from Boehringer. All other chemicals were of reagent grade quality.

Dimethyl sulfoxide, dimethoxyethane, acetonitrile, and triethylamine were dried on 3-Å molecular sieves and kept over calcium hydride.

Methods

Biological Preparations. Beef heart mitochondria were isolated by the method of Smith (1967). MgATP submitochondrial particles were obtained by sonication of beef heart mitochondria as described by Löw & Vallin (1963). Beef heart mitochondrial F_1-ATPase was prepared and stored as an ammonium sulfate suspension as described by Knowles & Penefsky (1972). Molar concentrations of purified F_1-ATPase are based on a molecular weight of 360000 (Lambeth et al., 1971). Before use, the suspension was centrifuged and the pellet solubilized with buffered sucrose (0.25 M sucrose and 50 mM Tris–acetate, pH 7.5) to a concentration of 5 mg of protein/mL; this solution was desalted by the elution–centrifugation method described by Penefsky (1977). A 1-mL syringe equipped with a porous polyethylene disk was filled with 1 mL of Sephadex G-50 fine equilibrated with the above buffer. The packed syringe was placed in a centrifuge tube and spun at low speed in an International table-top centrifuge to remove excess buffer. A sample of the ATPase solution

† From the Laboratoire de Biochimie (CNRS/ERA no. 36 et INSERM U. 191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble, and Faculté de Médecine de Grenoble, 38041 Grenoble Cedex, France. *Received February 26, 1980.* This investigation was supported in part by research grants from the "Fondation pour la Recherche Médicale" and the "Délégation Générale à la Recherche Scientifique et Technique".

[1] Abbreviations used: F_1-ATPase, beef heart mitochondrial ATPase; P_i, inorganic phosphate; ANPP, 4-azido-2-nitrophenyl phosphate; ANP, 4-azido-2-nitrophenol; NEM, *N*-ethylmaleimide; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; TMMg, 50 mM Tris, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, and 1 mM $MgSO_4$, pH 7.5; TMES, 50 mM Tris, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM EDTA, and 1 mM Na_2SO_4, pH 7.5.

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was applied to the top of the column. The pass-through solution obtained after centrifugation contained the desalted ATPase.

$^{32}P_i$ Binding Assays. Binding of P_i to F_1-ATPase was measured essentially as described by Penefsky (1977) and Kasahara & Penefsky (1978). The reaction mixture contained 70 μL of TMMg (or TMES) and 10 μL of $^{32}P_i$ solution. The binding reaction was initiated by addition of F_1-ATPase (20 μL) (to a final concentration of 1 mg of F_1-ATPase/mL) prepared as described above. After 20–25 min of incubation at room temperature the bound $^{32}P_i$ was separated from free $^{32}P_i$ by the elution–centrifugation method, as detailed in the above section, except that in the present case the Sephadex column was equilibrated with the TMMg or TMES buffer. After incubation with $^{32}P_i$, F_1-ATPase was applied to the top of the column. The eluate after centrifugation contained the $^{32}P_i$-bound ATPase.

[$^{32}P_i$]ANPP Binding Assays. Equilibrium binding in the dark was carried out as described for $^{32}P_i$. Aliquots of [$^{32}P_i$]ANPP (stored as a methanolic solution at −20 °C) were dried under nitrogen prior to solubilization in the required buffer.

In photoactivation assays, F_1-ATPase was preincubated in the dark with [^{32}P]ANPP for 20–25 min at 25 °C prior to photoirradiation. The sample was then pipetted into a 5-mL stoppered glass tube which was rotated nearly horizontally at 10 cm from the light source (250-W Osram halogen lamp) for 30 min. The tube was partially immersed in a bath maintained at 22–25 °C. A glass plate was placed between the light source and the glass tube to eliminate UV light which might be deleterious for the enzyme. After irradiation, the ATPase activity was determined. It was verified that F_1-ATPase irradiated under the same conditions, in the absence of ANPP, was not affected. The sample was then incubated with 10 mM P_i for 10 min at 25 °C, and the photolabeled F_1-ATPase was recovered in the excluded fraction obtained by elution–centrifugation (Penefsky, 1977). The excluded fraction was assayed for bound radioactivity and protein content. The [^{32}P]ANPP-labeled enzyme was incubated with a large excess of P_i prior to the final step of enzyme recovery to dilute $^{32}P_i$ released by photohydrolysis of [^{32}P]ANPP (see Results) and to reduce to a negligible value the contribution of $^{32}P_i$ to bound radioactivity.

ATPase Assay. Measurement of 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 solution (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.

Protein Determination. The protein concentration of mitochondria and submitochondrial particles was determined as described by Gornall et al. (1949). F_1-ATPase protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Radioactivity Determination. Radioactivity measurements were performed with an Intertechnique SL 30 liquid scintillation counter. Aqueous solutions (1-mL fractions) were counted in 10 mL of a scintillation fluid (Patterson & Greene, 1965).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ slab gel electrophoresis was performed in 10.3% polyacrylamide supplemented with 0.5% linear polyacrylamide to prevent cracking of the gels during drying (Zinker & Warner, 1976) and 0.1% $NaDodSO_4$ (Weber & Osborn, 1969). Urea slab gel electrophoresis was carried out in 5% polyacrylamide with 8 M urea as described by Knowles & Penefsky (1972). Radioautography was performed according to Bonner & Laskey (1974).

Synthesis and Properties of ANP. The starting material was 4-amino-2-nitrophenol (Aldrich). Its purity was checked by thin-layer chromatography on silica gel plates (Merck, F254) developed with $CHCl_3$/acetone/cyclohexane (50:30:20), R_f 0.5. An impurity, probably dinitroaminophenol (R_f 0.3), was occasionally found in some batches of 4-amino-2-nitrophenol. Accordingly, the compound was purified by chromatography on a silica gel column with the same solvent phase as that used for the silica gel plates.

4-Amino-2-nitrophenol (15.4 g) was dissolved in 100 mL of 1 N HCl at 50 °C and filtered through glass wool to eliminate any insoluble residue. After the filtrate was cooled at 0–5 °C, 25 mL of 12.5 N HCl was added. Then, an aqueous solution of $NaNO_2$ (7.5 g in 25 mL of water) was added dropwise with stirring. The temperature was kept at −10 °C by using a mixture of methanol and ice. All further reactions were carried out in the dark at 0 °C. One hundred milliliters of ethanol was first added, followed by 25 mL of a 24% (w/v) solution of NaN_3. After the mixture was stirred for 30 min, ethanol was removed by evaporation under vacuum and the remaining aqueous solution was extracted 3 times with 100 mL of diethyl ether each time. The combined ethereal extracts were washed with 100 mL of water, dried over Na_2SO_4 for 1 h, filtered, and evaporated to dryness under vacuum. The residue was dissolved in a minimum volume of petroleum ether (60–80 °C) and allowed to stand at −20 °C for crystallization. The yield of crystallized ANP was 70%. Thin-layer chromatography on silica gel plates in $CHCl_3$/acetone/cyclohexane, 5:3:2, gave a single yellow spot, with a R_f of 0.62; upon illumination the color of the spot changed to dark orange. ANP was characterized by its physical properties. NMR spectra of ANP in acetone-d_6 were taken with a 250-MHz Cameca spectrometer. ^{1}H NMR spectra gave the following data: δ (ppm from internal tetramethylsilane) 10.34 (s, 1 H, phenolic OH), 7.74 (d, 1 H, benzene 3 H), 7.46 (q, 1 H, benzene 5 H), 7.27 (d, 1 H, benzene 6 H); $J_{6-5} = J_{5-6} = 9$ Hz, $J_{3-5} = J_{5-3} = 3$ Hz. ^{13}C NMR spectra of ANP under conditions of hydrogen uncoupling showed three major peaks (C_3, C_6, and C_5) at 85.4, 92.6, and 99.5 ppm and three minor peaks (C_1, C_2, and C_4) at 103.5, 105.4, and 122.7 ppm (ppm from acetone-d_6). Mass spectra of ANP were taken with a GEC-AE MSQ apparatus. Three main peaks at mass/charge (m/e) ratios of 180 (molecular peak), 152 (minus N_2), and 154 were obtained. IR (KBr) spectra showed the azido band at 2100 cm^{-1}. UV spectra were characterized by maxima at 247 and 386 nm in methanol, at 260 and 458 nm in 10 mM NaP_i, pH 8.0, and at 250 and 394 nm in 10 mM HCl.

Synthesis of ANPP. Two methods were utilized to phosphorylate ANP into ANPP. One was based on the use of P_i in the presence of trichloroacetonitrile, and the other involved $POCl_3$. The first one was found to be more convenient for synthesis of [^{32}P]ANPP.

(1) Phosphorylation by Phosphate in the Presence of Trichloroacetonitrile. The method was similar to that originally described by Cramer & Weimann (1960) and subsequently used by Symons (1968) for synthesis of nucleoside monophosphate. A solution of 10 mCi of $^{32}P_i$ was diluted with 45 μmol of phosphoric acid in 3 mL of acetonitrile. The

mixture was evaporated to dryness, the residue was taken up in acetonitrile, and acetonitrile was again removed by evaporation. This procedure was repeated 3 times. Then a solution of ANP, 200 mg in 2 mL of acetonitrile, was added. After evaporation of the solvent, 3 mL of dimethyl sulfoxide was added, followed by 125 μL of triethylamine and 110 μL of trichloroacetonitrile with stirring. After 1 h at 37 °C, 1 mL of water was added and the mixture was incubated for an additional 1 h and then dried under vacuum. The residue was redissolved in methanol and evaporated to dryness under reduced pressure. This procedure was repeated 3 times. The dried residue was dissolved in 5 mL of water and extracted with diethyl ether (3 × 10 mL). The water phase was evaporated to dryness; the residue was dissolved in 1 mL of methanol and applied as bands 15 cm wide to four silica gel analytical TLC plates 20 cm in width (Merck F254). The plates were developed with a mixture of ethanol and 1 M ammonium acetate (7:3 v/v). A major band was quickly localized under UV light; the material was scraped off and extracted with methanol. After centrifugation, the methanol extract was purified by descending chromatography on Whatman 1 paper in ethanol and 1 M ammonium acetate (7:3). A narrow band of the dried chromatogram was cut out and illuminated with light to detect ANPP. The corresponding spot (R_f ~0.36) was cut out and eluted with methanol.

(*2*) *Phosphorylation by $POCl_3$.* This reaction was carried according to the procedure described by Maassen & Möller (1974, 1977) for the synthesis of 4-azidophenyl phosphate. [^{32}P]$POCl_3$ (10 mCi, 55.6 mg) was allowed to react with 2 mL of dimethoxyethane containing 130 mg of 1,4-diazabicyclo[2.2.2]octane for 15 min; then a solution of ANP, 65 mg in 2 mL of dimethoxyethane, was added to the stirred mixture. After reaction overnight at room temperature, the solvent was removed by rotary evaporation and then the residue was treated with 5 mL of cold water and chromatographed on a Dowex 50 WX8 column (1.5 × 10 cm) equilibrated with 10 mM HCl. Elution was carried out with 10 mM HCl, and the collected fractions (1 mL) were analyzed by thin-layer chromatography (see above). Fractions giving one spot with an R_f corresponding to ANPP were pooled and evaporated under vacuum. The residue was dissolved in 5 mL of water and extracted twice with 10 mL of diethyl ether. After removal of water by rotary evaporation, the residue was dissolved in 1 mL of methanol and dried under vacuum. This procedure was repeated 3 times. Then the methanolic solution was chromatographed on four analytical silica gel plates with a solvent made of 1 M ammonium acetate and ethanol (3:7 v/v). The main UV band was located by rapid UV illumination and then scraped off. After extraction with methanol, the material was submitted to a final chromatography on a sheet of Whatman 1 paper as described above.

Hydrolysis of ANPP by 1 M NaOH at 100 °C yielded equivalent molar amounts of P_i, as measured according to Fiske & SubbaRow (1925), and ANP, as measured by UV spectroscopy. Alkaline phosphatase was also able to hydrolyze ANPP in P_i and ANP (see Results).

UV spectra of ANPP in methanol showed maxima at 247 and 335 nm with ϵ_{335} = 1810 M^{-1} cm^{-1} and $\epsilon_{247}/\epsilon_{335}$ = 9.2 (Figure 1A, trace 1).

Results

Photodecomposition of ANPP. The effect of high-intensity light flashes on the spectrum of ANPP is illustrated in Figure 1A. The decrease in absorbance at 250 nm was accompanied by a progressive increase of the optical density at 370–400 nm. In contrast, photoirradiation of ANP led essentially to a decrease in the 250-nm region. This suggests that photoirradiation of ANPP results in photoproducts in which the aromatic ring has been substantially modified and that only a small amount of ANP accumulates.

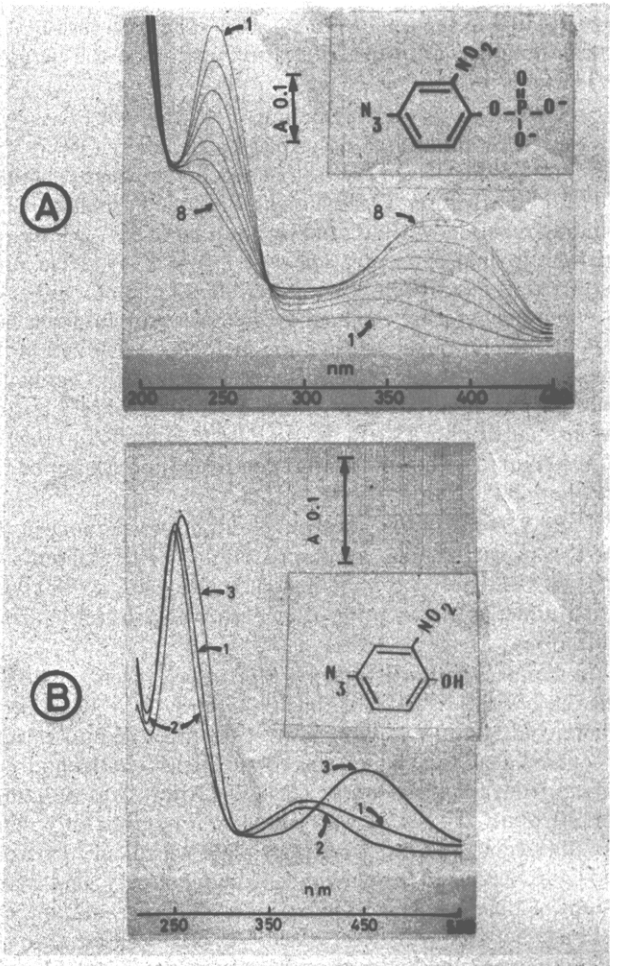

FIGURE 1: Absorption spectra of ANPP and ANP. Photoreactivity of ANPP. (A) A solution of ANPP (33 μM) in methanol in a 3-mL quartz cuvette of 1-cm pathway was submitted to successive flashes of light delivered by an Ultrablitz-Matador III GMBH generator. The lamp was placed 5 cm from the cuvette. Trace 1, control; trace 2, 3 flashes; trace 3, 6 flashes; trace 4, 9 flashes; trace 5, 12 flashes; trace 6, 20 flashes; trace 7, 28 flashes; trace 8, 38 flashes. (B) For comparison with ANPP, the spectrum of ANP (20 μM) in methanol is shown in trace 1. Spectra 2 and 3 are those of ANP (20 μM) in 10 mM HCl and 10 mM NaP_i, pH 8, respectively.

Photoirradiation of ANPP was accompanied by the release of P_i from ANPP; photoinduced release of P_i was followed under the same conditions of continuous irradiation by an Osram lamp as those used for photoinactivation of F_1-ATPase (Figure 2). $^{32}P_i$ released into the medium was separated from [^{32}P]ANPP by ascending chromatography on Whatman 1 paper in formic acid/diisopropyl ether/H_2O (90:60:3). The spots corresponding to $^{32}P_i$ (R_f 0.65) and [^{32}P]ANPP (R_f 0.80) were revealed by autoradiography and cut out, and their radioactivity was counted. The time course of the light-induced [$^{32}P_i$]ANPP hydrolysis is shown in Figure 3; after 30 min of illumination, which was the period of irradiation used in routine photolabeling of F_1-ATPase, about 50% of ANPP was photohydrolyzed. These data were corroborated by direct chemical determination of P_i present in the medium before and after illumination. The marked photohydrolysis of ANPP is noteworthy and should be remembered in the interpretation of the photoinactivation data obtained with F_1-ATPase.

Interaction between ANPP and F_1-ATPase in the Dark. Competitive Inhibition of $^{32}P_i$ Binding by ANPP. In agree-

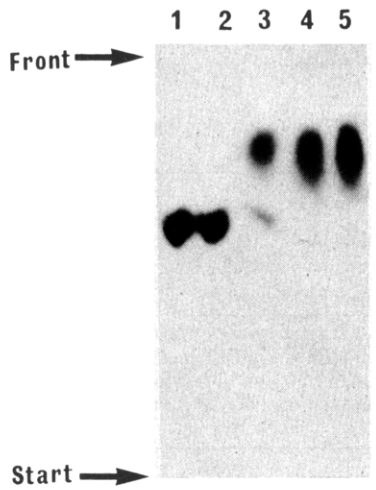

FIGURE 2: Stability of [^{32}P]ANPP. Analysis by ascending chromatography on Whatman 1 paper in diisopropyl ether/98% formic acid/H_2O (90:60:3 v/v/v). The different tracks correspond to the following compounds and conditions: (1) $^{32}P_i$; (2) [^{32}P]ANPP incubated with alkaline phosphatase for 30 min at 20 °C; (3) [^{32}P]-ANPP photoirradiated for 20 min at 20 °C; (4) [^{32}P]ANPP incubated with F_1-ATPase for 30 min at 20 °C in TMMg buffer; (5) [^{32}P]ANPP control.

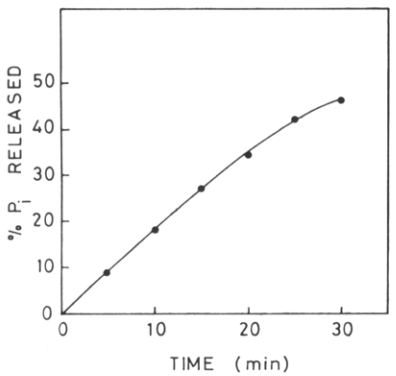

FIGURE 3: Time course of light-induced ANPP hydrolysis. [^{32}P]ANPP (100 μM) in water was photoirradiated for various periods of time with an Osram lamp as described under Methods. Aliquots (5 μL) were then chromatographed on Whatman 1 paper in diisopropyl ether/98% formic acid/H_2O (90:60:3 v/v/v). After autoradiography (see Figure 2), the radioactive spots were cut off and the radioactivity was measured.

ment with Penefsky (1977) and Kasahara & Penefsky (1977, 1978), we found that F_1-ATPase exhibits a single binding site for $^{32}P_i$ in the presence of Mg^{2+} (TMMg buffer; K_d = 37 μM); virtually no binding was observed when Mg^{2+} was replaced by EDTA (less than 0.01 mol of $^{32}P_i$ per mol of F_1-ATPase in TMES buffer). In these experiments bound $^{32}P_i$ was separated from free $^{32}P_i$ by rapid filtration on a small Sephadex column as described by Penefsky (1977).

Similar binding experiments, carried out in the dark with [^{32}P]ANPP as a ligand of F_1-ATPase instead of $^{32}P_i$, were hampered by the strong interaction which exists between the Sephadex gel and phenol derivatives [see Brook & Munday (1970) and Determan & Walter (1968)]. It was, however, possible to demonstrate that ANPP interacts with the phosphate site of the ATPase by examination of the inhibitory effect of ANPP on $^{32}P_i$ binding by F_1-ATPase. As shown in Figure 4, ANPP was a potent inhibitor of $^{32}P_i$ binding by F_1-ATPase; the K_i value was as low as 60 μM. The inhibition was competitive in nature, suggesting that P_i and ANPP are recognized by the same site. At a final concentration of 100 μM, ANP, the ANPP precursor, had no effect on $^{32}P_i$ binding. Additional assays in the dark indicated that ANPP does not

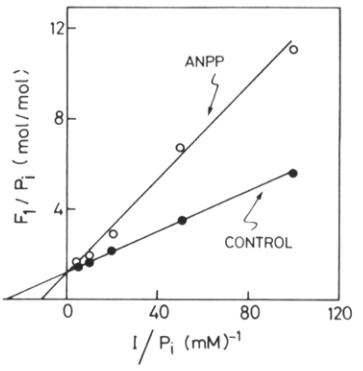

FIGURE 4: Competitive inhibition of $^{32}P_i$ binding by ANPP in the dark. F_1-ATPase (1 mg/mL) in TMMg buffer was incubated in the dark with (○) or without (●) ANPP (100 μM) and with various concentrations of $^{32}P_i$ ranging from 10 to 200 μM for 25 min at room temperature. $^{32}P_i$ binding was then determined as described under Methods. The K_i for ANPP was about 60 μM, and the K_d for $^{32}P_i$ was 37 μM.

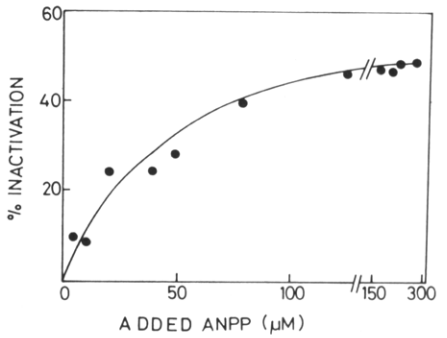

FIGURE 5: Photoinactivation of F_1-ATPase by various concentrations of ANPP. F_1-ATPase (1 mg/mL) was preincubated in the dark with various concentrations of ANPP up to 300 μM in TMMg buffer for 25 min and then photoirradiated for 30 min at 20 °C. The ATPase activity was measured at the end of the photoirradiation period as described under Methods.

significantly modify [^{14}C]ADP binding by F_1-ATPase (not shown). Finally, it must be noted that ANPP is not hydrolyzed by F_1-ATPase in the absence of light (Figure 2). In contrast, alkaline phosphatase was able to cleave the phosphate bond of ANPP (Figure 2).

Photoinactivation of F_1-ATPase by ANPP. Preincubation of F_1-ATPase with ANPP in the dark followed by photoirradiation resulted in loss of hydrolytic activity of the enzyme. The dark control (incubation with ANPP in the absence of light) and the light control (F_1-ATPase irradiated in the absence of ANPP) showed no significant change in the ATPase activity. As shown in Figure 5, the loss of activity upon photoirradiation depended on the concentration of ANPP up to 120 μM; the half-maximal effect was at 45 μM ANPP. A maximal loss of activity of 40–60% was attained after a 30-min irradiation under our test conditions (cf. Methods). It must be remembered that ANPP is very photosensitive and is hydrolyzed under light to release P_i and photoproducts. Light-induced decomposition of ANPP might therefore compete with photoinactivation of F_1-ATPase by ANPP. In addition, one cannot exclude that photoproducts may compete with ANPP for binding to the enzyme. The ATPase activity of photolabeled F_1 could not be restored after gel filtration, indicating an irreversible inactivation of the enzyme. The fraction of F_1-ATPase still active after photoirradiation with ANPP and the untreated F_1-ATPase had the same K_m. This indicates an all or none effect of photoinactivation by ANPP.

It was essential to check whether ANP, the precursor of ANPP, could inactivate F_1-ATPase upon light irradiation. No

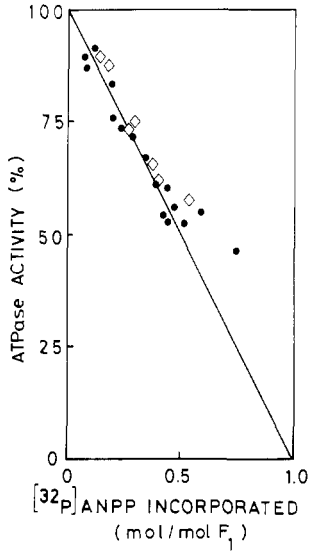

FIGURE 6: Correlation between photoinactivation of F_1-ATPase and covalent binding of [^{32}P]ANPP. Photoinactivation of F_1-ATPase was performed with various [^{32}P]ANPP concentrations up to 200 μM in TMMg buffer (●) and in TMES buffer (◇) as in Figure 5. After 25 min of preincubation in the dark followed by 30 min of photoirradiation at 20 °C, the ATPase activity, the bound radioactivity, and the protein content were determined as described under Experimental Section. The bound radioactivity was assumed to correspond to bound [^{32}P]ANPP.

significant inactivation of F_1-ATPase was found when the enzyme was photoirradiated with 100 μM ANP in TMMg or TMES buffer.

Photoaffinity Labeling of F_1-ATPase by [^{32}P]ANPP. Labeling Stoichiometry and Effect of P_i. Photoinactivation of F_1-ATPase by [^{32}P]ANPP was accompanied by covalent binding of radioactivity which was recovered with the inactivated enzyme after gel filtration. When a further 30-min irradiation was carried out on the gel filtrate, no further change of enzyme activity was observed, and interestingly, no release of the bound radioactivity could be detected. On the assumption that the whole molecule of [^{32}P]ANPP bound to F_1-ATPase, the light stability of bound ANPP clearly contrasts with the light sensitivity of free ANPP.

Investigation of the binding stoichiometry was carried out after separation of the covalently photolabeled complex by the elution centrifugation method of Penefsky (1977). A linear relationship between the covalent radiolabeling and the loss of ATPase activity was found (Figure 6). By extrapolation, it could be calculated that complete inactivation of F_1-ATPase was accompanied by incorporation of ^{32}P radioactivity corresponding to 1 mol of [^{32}P]ANPP per mol of F_1-ATPase. The same stoichiometry was obtained whether photoirradiation of F_1-ATPase was performed in TMMg or TMES buffer.

P_i added to F_1-ATPase prior to photoirradiation markedly protected the enzyme against photoinactivation by ANPP, and it prevented the covalent binding of [^{32}P]ANPP (Table I). Although the above data clearly pointed to the reaction of [^{32}P]ANPP with the phosphate site of F_1-ATPase, they gave no information on the nature of the bound radioactivity, which could correspond to either the whole molecule of [^{32}P]ANPP or the ^{32}P moiety of [^{32}P]ANPP. In an attempt to clarify this point, F_1-ATPase was photoirradiated with ANPP and then passed through a Sephadex column to remove free ANPP and photoproducts. Comparison of the spectrum of photolabeled F_1-ATPase with that of the nonmodified enzyme was consistent with a significant binding to the photoirradiated enzyme of photoproducts arising from ANPP. However, the amount of bound photoproducts was difficult to quantify, and it is possible that some P_i could have been discharged from ANPP to phosphorylate an amino acid residue(s) at the phosphate site of the enzyme.

Table I: Effect of P_i on Inactivation and Covalent Binding of [^{32}P]ANPP to F_1-ATPase[a]

buffer used	P_i (mM)	% remaining act.	bound [^{32}P]ANPP (mol/mol of F_1-ATPase)
TMMg	0	56	0.47
	1	83	0.18
	10	88	0.06
TMES	0	64	0.41
	1	73	0.26

[a] F_1-ATPase (1 mg/mL) was preincubated at 20 °C for 20 min in the dark with 100 μM [^{32}P]ANPP in TMMg or TMES buffer with or without P_i. After 30 min of photoirradiation, ATPase activity was measured and radioactivity was determined as described under Methods.

Identification of the Photolabeled Subunit in F_1-ATPase. After photoirradiation in the presence of [^{32}P]ANPP, yielding approximately 50% inactivation, F_1-ATPase was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis according to Weber & Osborn (1969). The α and γ subunits of F_1-ATPase were identified by [^{14}C]NEM labeling (Senior, 1975), and the β subunit was identified by [^{14}C]DCCD labeling (Pougeois et al., 1979). By this procedure, [^{32}P]ANPP was found to be located in subunit β (Figure 7A).

Separation of the α and β subunits of F_1-ATPase was also carried out by polyacrylamide gel electrophoresis in the presence of 8 M urea according to Knowles & Penefsky (1972) (Figure 7B). Here again, [^{32}P]ANPP was clearly located in the β subunit. Subunits α and γ were completely free of [^{32}P]ANPP.

Photolabeling of Submitochondrial Particles by [^{32}P]-ANPP. F_1-ATPase in inside-out particles obtained by sonication of beef heart mitochondria is exposed to the outer medium (Racker, 1970); it must therefore be accessible to ANPP. Sonicated particles were photoirradiated in the presence of [^{32}P]ANPP as described in the legend to Figure 8 and lysed by $NaDodSO_4$. The lysate was then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The α, γ, and ϵ subunits of [^{14}C]NEM-labeled F_1-ATPase were used as marker peptides for estimation of molecular weights. Autoradiography revealed two main labeled bands with apparent molecular weights close to 30 000 and 50 000. The latter one most probably corresponded to the β subunit of F_1-ATPase. A very faint band with a molecular weight of 53 000–55 000 was also apparent; it may belong to the α subunit of bound ATPase. It must, however, be noted that subunit α in purified F_1-ATPase was not photolabeled by ANPP. The 30 000 molecular weight protein remains to be identified. A likely candidate is the phosphate carrier.

Discussion

The synthesis of a new photoaffinity label, ANPP, in its radioactive form, [^{32}P]ANPP, is described in the present paper. The fact that ANPP reacts with the P_i site of F_1-ATPase is based on the following findings. ANPP competes, in the absence of photoirradiation, with the binding of $^{32}P_i$ to F_1-ATPase, its binding affinity being similar to that of P_i. Upon photoirradiation, ANPP inactivates F_1-ATPase, in parallel with covalent binding to the enzyme. Protection against photoinactivation and photolabeling by ANPP is afforded by

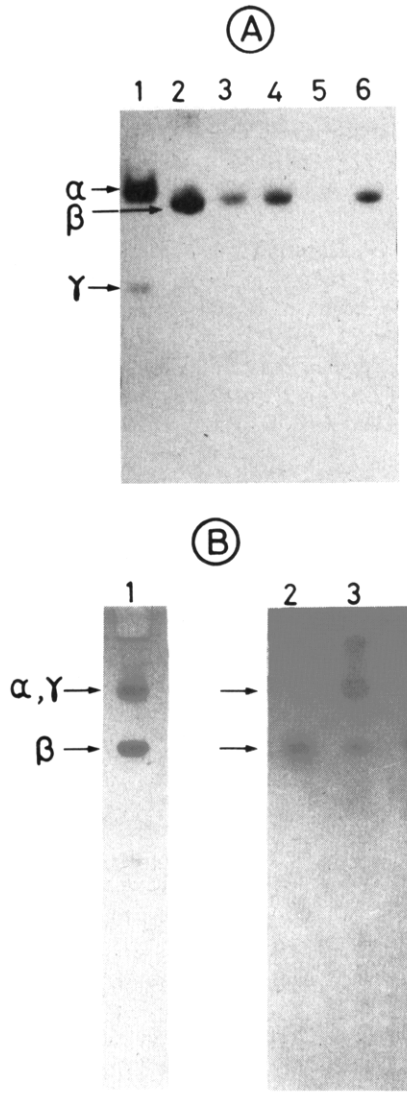

FIGURE 7: Photolabeling of F_1-ATPase with [^{32}P]ANPP. Analysis of labeled peptides by $NaDodSO_4$–polyacrylamide (A) or urea–polyacrylamide (B) gel electrophoresis followed by autoradiography. (A) F_1-ATPase was labeled as described under Methods in TMMg buffer with 50 μM [^{32}P]ANPP (track 3), 150 μM [^{32}P]ANPP (track 4), and 150 μM [^{32}P]ANPP plus 10 mM P_i (track 5) or in TMES buffer with 100 μM [^{32}P]ANPP (track 6). Identical aliquots (5 μg) of the different enzyme samples were subjected to $NaDodSO_4$ gel electrophoresis. Tracks 1 and 2 correspond to the migration of labeled marker peptides, which were subunits α, γ, and ϵ of F_1-ATPase labeled by [^{14}C]NEM according to Senior (1975) (track 1) and subunit β of F_1-ATPase labeled by [^{14}C]DCCD according to Pougeois et al. (1979). (B) Track 2 corresponds to the migration of 5 μg of F_1-ATPase labeled by 150 μM [^{32}P]ANPP. Tracks 3 and 4 correspond to the migration of labeled marker peptides: track 3, F_1-ATPase labeled by 4 mM [^{14}C]NEM in 8 M urea for 30 min [note the labeling by [^{14}C]NEM of subunit β of F_1-ATPase denatured by urea]; track 4, F_1-ATPase labeled by [^{14}C]DCCD (Pougeois et al., 1979). In track 1 of series B, F_1-ATPase subunits were revealed by Coomassie Blue staining.

preincubation of F_1-ATPase with P_i. The nonphosphorylated precursor, ANP, has none of the above properties. All these data indicate that ANPP meets the requirements for competent photoaffinity labeling and that, in particular, ANPP recognizes F_1-ATPase at the same site as P_i. In photoaffinity labeling experiments, the phenomenon of pseudophotoaffinity represents a complication (Ruoho et al., 1973); all experiments described here were carried out in the presence of Tris buffer, which scavenges the freely migrating reactive species. By the labeling of F_1-ATPase with [^{32}P]ANPP, it has been demonstrated that subunit β contains the P_i binding site. This is

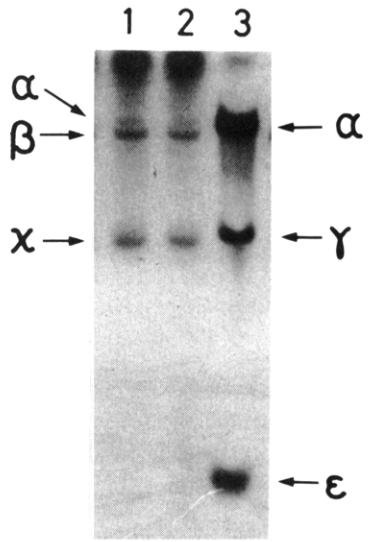

FIGURE 8: Photolabeling of sonicated particles from beef heart mitochondria in the presence of [^{32}P]ANPP. Analysis of labeled peptides by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography. [^{32}P]ANPP (final concentration 100 μM) was preincubated for 20 min at 0 °C in the dark with MgATP particles (0.4 mg of protein) in 0.1 mL of 0.25 M sucrose, 10 mM Tris–acetate, pH 7.5, 1 mM NaF, and 1 mM $MgCl_2$. NaF was added to inhibit potential phosphatase activity (Krisch, 1971). Then the sample was photoirradiated for 30 min in ice as described under Methods. The particles were lysed with 3% $NaDodSO_4$ and 3% β-mercaptoethanol and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (tracks 1 and 2). Track 3 corresponds to the migration of marker peptides which were subunits α (M_r 55 000), γ (M_r 33 000), and ϵ (M_r 7500) of F_1-ATPase labeled with [^{14}C]NEM according to Senior (1975).

consistent with the view that subunit β in F_1-ATPase is also capable of binding ADP and ATP [for review, cf. Senior (1979)] and that it most likely possesses the catalytic site of F_1-ATPase. At this point, it might be appropriate to discuss the nature of the labeling of F_1-ATPase after irradiation in the presence of [^{32}P]ANPP, especially in view of the photohydrolysis of ANPP. It is clear that, upon photoirradiation, ^{32}P radioactivity was covalently bound to F_1-ATPase. However, it had to be clarified whether the bound radioactivity reflected the binding of the whole [^{32}P]ANPP molecule or only the [^{32}P]phosphate moiety of [^{32}P]ANPP. Although spectral data afforded evidence for binding of at least a fraction of added ANPP to F_1-ATPase, it could not be excluded that, upon irradiation, some bound ANPP discharged its P_i to phosphorylate an amino acid residue(s) in the vicinity of the P_i binding site in F_1-ATPase; this artificial photophosphorylation of an amino acid residue(s) would be consistent with the resistance of the bound ANPP to photohydrolysis which contrasted with the photohydrolysis of free ANPP.

Careful studies of P_i binding to purified beef heart F_1-ATPase by Penefsky (1977) and Kasahara & Penefsky (1977, 1978) pointed to the occurrence of two types of P_i binding sites, a high-affinity saturable binding site with a K_d of 37 μM at pH 7.5 and a second low-affinity nonsaturable site. The high-affinity binding site was capable of binding 1 mol of P_i per mol of F_1-ATPase. Our present data with [^{32}P]ANPP are in general accordance with these specific properties of P_i binding; in fact the K_i value for ANPP, 60 μM, is close to the K_d value for the high-affinity binding of P_i (37 μM) and complete inactivation corresponds to the binding of 1 residue of ANPP to 1 mol of F_1-ATPase. Thus, ANPP binding mimics the high-affinity binding of P_i to F_1-ATPase. There is, however, a minor difference which concerns the effect of EDTA and divalent cations on P_i binding. Penefsky (1977)

reported that divalent cations are required for P_i binding; no P_i binding could be detected in the presence of EDTA. In contrast, the amount of bound ANPP was similar in the presence of Mg^{2+} or EDTA. It is possible that the P_i site of F_1-ATPase can recognize P_i in the presence of EDTA; but the enzyme has too low of an affinity for P_i to retain it after Sephadex exclusion chromatogaphy. This difference in affinity is not observed in the case of ANPP, probably because ANPP, under light, binds irreversibly to F_1-ATPase.

The instability of the phosphate bond of ANPP in light indicates that ANPP is analogous, in this respect, to 2-nitrobenzyl phosphate and 1-(2-nitrophenyl)ethyl phosphate, which were designated as caged P_i in a recent report by Kaplan et al. (1978). In our experiments that were performed essentially with soluble F_1-ATPase, the photodependent release of P_i from ANPP was most likely responsible for the limited inactivation of F_1-ATPase, the upper attainable value being 60% of the expected maximum.

Labeling experiments of submitochondrial particles by [^{32}P]ANPP showed two main radioactive bands of approximately 50 000 and 30 000 daltons and a slightly labeled band of 53 000–55 000 daltons. It is remarkable that, in spite of the large number of protein species present in submitochondrial particles, only two peptides are capable of recognizing ANPP. The M_r 50 000 peptide most likely corresponds to the β subunit of F_1-ATPase. The M_r 30 000 peptide is another P_i binding component of the inner mitochondrial membrane. Experiments are presently being carried out to check whether this component could be the mitochondrial P_i carrier.

The photoaffinity label 2-azido-4-nitrophenol, an isomer of ANP, has been shown by Hanstein & Hatefi (1974) and Hanstein et al. (1979) to bind to two peptides of mitochondria, approximately 56 000 and 31 000 daltons. The former peptide was identified as subunit α of F_1-ATPase and the latter as the membrane portion of the oligomycin-sensitive ATPase complex. Kurup & Sanadi (1977) also investigated the photolabeling of submitochondrial particles by 2-azido-4-nitrophenol; they found labeled peptides with molecular weights of 55 000, 20 000, and 9000. We demonstrated unambiguously that ANPP binds essentially to the β subunit of purified F_1-ATPase and to a 30 000-dalton peptide. These data indicate that 2-azido-4-nitrophenol and ANPP have different binding properties.

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