Photoaffinity Labeling of Mitochondrial Adenosinetriphosphatase by 2-Azidoadenosine 5′-[α-^{32}P]Diphosphate†

François Boulay,* Pascal Dalbon, and Pierre V. Vignais

Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble, 38041 Grenoble Cedex, France

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ABSTRACT: 2-Azidoadenosine 5′-diphosphate (2-azido-ADP) labeled with ^{32}P in the α-position was prepared and used to photolabel the nucleotide binding sites of beef heart mitochondrial F_1-ATPase. The native F_1 prepared by the procedure of Knowles and Penefsky [Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6617–6623] contained an average of 2.9 mol of tightly bound ADP plus ATP per mole of enzyme. Short-term incubation of F_1 with micromolar concentrations of [α-^{32}P]-2-azido-ADP in the dark in a Mg^{2+}-supplemented medium resulted in the rapid supplementary binding of 3 mol of label/mol of F_1, consistent with the presence of six nucleotide binding sites per F_1. The K_d relative to the reversible binding of [α-^{32}P]-2-azido-ADP to mitochondrial F_1 in the dark was 5 μM in the presence of $MgCl_2$ and 30 μM in the presence of ethylenediaminetetraacetic acid. A linear relationship between the percentage of inactivation of F_1 and the extent of covalent photolabeling by [α-^{32}P]-2-azido-ADP was observed for percentages of inactivation up to 90%, extrapolating to 2 mol of covalently bound [α-^{32}P]-2-azido-ADP/mol of F_1. Under these conditions, only the β subunit was photolabeled. Covalent binding of one photolabel per β subunit was ascertained by electrophoretic separation of labeled and unlabeled β subunits based on charge differences and by mapping studies showing one major radioactive peptide segment per photolabeled β subunit. Upon incubation for several hours at room temperature in the dark with saturating concentrations of [α-^{32}P]-2-azido-ADP, the amount of bound radioactivity increased steadily, and at 20 h, it amounted to 4.3 mol/mol of F_1. This slow binding was accompanied by release of endogenous ADP or ATP, so that the total amount of bound nucleotides never exceeded 6 mol/mol of F_1. The slowly reacting [α-^{32}P]-2-azido-ADP was found to bind covalently, upon photoirradiation, to the α and β subunits, with net predominance for the β subunits possibly because of the orientation of the probe. Mapping studies indicated different patterns of photolabeling of the β subunit after short and long periods of preincubation with [α-^{32}P]-2-azido-ADP. These results are compatible with the presence of three loose nucleotide binding sites and three tight nucleotide binding sites per mitochondrial F_1; the loose sites would be located on the β subunits and the tight sites at the junction of the α and β subunits.

It is widely accepted that, of the five types of subunits that compose the catalytic F_1[1] sector of the mitochondrial, bacterial, and chloroplast H^+-ATPases, only the α and β subunits contain nucleotide binding sites. F_1 contains six nucleotide binding sites, these sites being distributed between the three α and the three β subunits [for review, see Vignais & Satre (1984)]. Photoactivable derivatives of ATP or ADP have proven to be useful in this context, especially to localize the nucleotide sites in the α and β subunits. Because of its structural features, the recently introduced 2-azido-ADP appears particularly attractive for mapping studies: the azido group is placed directly on the purine ring, resulting in an "anti" conformation typical of the natural nucleotide. Photoirradiation of chloroplast F_1 (Czarnecki et al., 1982) and mitochondrial F_1 (Dalbon et al., 1985) with 2-azido-ADP resulted in the exclusive labeling of the β subunit. This is in contrast to 8-azido-ADP or -ATP (Wagenvoord et al., 1979, 1980), 3′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]ADP, -ATP, or -AMPPNP (Lunardi et al., 1977, 1981, 1982), 3′-*O*-[3-(4-azido-2-nitrophenyl)propionyl]ADP (Weber et al., 1985), 3′-*O*-(5-azidonaphthoyl)ADP (Lübben et al., 1984), and 3′-*O*-(4-benzoylbenzoyl)ATP (Williams & Coleman, 1982), which were found to photolabel both the α and β subunits of mitochondrial F_1. The intriguing photolabeling specificity of 2-azido-ADP for the β subunit warranted further exploration of the interaction of this probe with F_1. The aim of the present study was to explore in more detail the binding parameters of 2-azido-ADP with respect to mitochondrial F_1. The radiolabeled 2-azido-ADP used in this work was labeled in the α-position by ^{32}P to avoid loss of radioactivity during the chemical processing of photolabeled F_1.

Materials and Methods

Chemicals. 2-Chloroadenosine was from Sigma, *p*-toluenesulfonic acid and ethyl orthoformate were from Aldrich, and trichloroacetonitrile was from Fluka. $H_3{}^{32}PO_4$ and EN^{3}Hance were purchased from New England Nuclear. Protease V8 was from Miles, cyanogen bromide was from Merck, acrylamide and *N*,*N*′-methylenebis(acrylamide) were from Eastman Kodak, PEI–cellulose F, cellulose F, silica gel 60F on aluminum plates were from Merck, linear acrylamide

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[1] Abbreviations: F_1, soluble coupling factor 1; $NaDodSO_4$, sodium dodecyl sulfate; PEI–cellulose, poly(ethylenimine)–cellulose; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; HPLC, high-performance liquid chromatography; TMMg, 50 mM Tris, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, and 2 mM $MgCl_2$, pH 7.5; TMES, 50 mM Tris, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, and 1 mM EDTA, pH 7.5; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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was from BDH, and Sephadex G-50 fine grade was from Pharmacia. All reagents used were of the purest grade commercially available.

Preparation of Mitochondrial F_1. F_1 was prepared from beef heart mitochondria and stored as a precipitate in ammonium sulfate in the presence of 4 mM ATP (Knowles & Penefsky, 1972). Before use, an aliquot sample was centrifuged, and the pellet was dissolved in 0.25 M sucrose–50 mM Tris–acetate at pH 7.5 and desalted by centrifugation–filtration, in a 1-mL syringe filled with 1 mL of Sephadex G-50 fine grade equilibrated in the same buffer (Penefsky, 1977). Molar concentrations of purified F_1 were calculated on the basis of a M_r of 360000 (Lambeth et al., 1971). The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as standard ($\epsilon^{1\%}_{279nm} = 6.67$).

Assay of ATPase Activity. ATPase activity of F_1 was measured at 30 °C. The reaction medium, in a final volume of 0.5 mL, contained 40 mM Tris-HCl, 10 mM ATP, 5 mM $MgCl_2$, 10 μg of pyruvate kinase, and 2 mM phosphoenolpyruvate, final pH 8.0. The reaction was started by addition of an aliquot fraction of the F_1 solution (3–5 μg) and stopped after 2 min by addition of 0.2 mL of ice-cold 50% (v/v) trichloroacetic acid. The released P_i was determined by the method of Fiske & Subbarow (1925). At low concentrations of ATP, the ATPase activity was determined spectrophotometrically. The reaction mixture in 2 mL contained 50 mM Tris-HCl, 240 mM sucrose, 0.2 mM NADH, 1 mM free magnesium (as $MgCl_2$), 2 mM phosphoenolpyruvate, 50 μg of pyruvate kinase, 25 μg of lactate dehydrogenase, 10 mM KCl, 10 mM $NaHCO_3$ (final pH 8.0), and different concentrations of Mg-ATP. The reaction was monitored by following the oxidation of NADH at 340 nm.

Radioactivity Determination. Aqueous radioactive solutions (1.0-mL fractions) were dispersed in 10 mL of a scintillation fluid (Patterson & Greene, 1965). Radioactivity was measured by liquid scintillation counting.

Polyacrylamide Gel Electrophoresis. For $NaDodSO_4$ slab gel electrophoresis, the 20% polyacrylamide gels were supplemented with 0.5% linear polyacrylamide to prevent cracking of the gels during drying (Zinker & Warner, 1976). The 20% polyacrylamide gel was topped by a 10% polyacrylamide stacking gel. The buffers were prepared as described by Cabral & Schatz (1979).

Urea slab gel electrophoresis was carried out in 5% polyacrylamide with 8 M urea for 6 h under 60 mA, following the procedure of Knowles & Penefsky (1972) with the following modifications: the pH of the gel was lowered to 8.5, and the pH of the electrode buffer, composed of 56 g of glycine and 3 g of Tris base per liter was adjusted to 8.0 with HCl. Autoradiography was performed after impregnation of the gel with EN^{3}Hance (Laskey & Mills, 1975). The gels were dried and exposed for several hours to a Fuji RX film at −70 °C with a Cronex intensifying screen.

Chemical and Enzymatic Fragmentation of Photolabeled F_1. Cleavage at methionyl residues by cyanogen bromide was performed as described by Gross (1967). Protease V8 from *Staphylococcus aureus* was incubated with 50-μg aliquots of F_1 in 50 mM ammonium bicarbonate for 1 h at 25 °C.

2-Azidoadenosine 5′-[α-^{32}P]Diphosphate Binding Assays. In the study of the reversible binding of 2-azido-ADP to F_1-ATPase, all the reactions were carried out in the dark. The reaction medium, final volume 0.1 mL, consisted of 50 mM Tris base, 50 mM MES, 50 mM sucrose supplemented with either 1 mM EDTA (TMES buffer) or 2 mM $MgCl_2$ (TMMg buffer), 40 μg of F_1, and increasing concentrations of [α-^{32}P]-2-azido-ADP; after 10 min of incubation at room temperature, the bound radioactivity was separated from free [α-^{32}P]-2-azido-ADP by the elution–centrifugation method of Penefsky (1977) with 1-mL Sephadex G-50 columns equilibrated with TMMg or TMES buffer, respectively. The binding stoichiometry was calculated on the basis of the protein content and the amount of radioactivity on aliquot samples.

In photoinactivation assays, F_1-ATPase was preincubated in the dark with [α-^{32}P]-2-azido-ADP for 10 min at room temperature prior to photoirradiation. The sample (100 μL) was then pipetted into the cap of an Eppendorf tube and irradiated for 3 s with a xenon lamp XBO 1000 W/HS equipped with a parabolic reflector and placed at 30 cm. The power supply SVX 1000 (Müller Gmb, Moosinning, West Germany) operated at 0.9 kW. The thermal energy of infrared radiation was evacuated by a circulating water filter of 10-cm thickness. Prolonged exposure of F_1 to UV light is deleterious to the enzyme; however, after 3-s photoirradiation, less than 5% of ATPase activity was lost in comparison to a nonirradiated control. The rate of photodestruction of the 2-azido-ADP under the above conditions of irradiation was determined; half of the 2-azido-ADP disappeared after 3 s of photoirradiation. This is compatible with efficient photolabeling of F_1-ATPase.

The F_1 sample photoirradiated with [α-^{32}P]-2-azido-ADP was incubated with 5 mM ADP for 15 min at 20–25 °C. The F_1 covalently bound [α-^{32}P]-2-azido-ADP was finally recovered in the excluded fraction after elution–centrifugation through Sephadex G-50 (Penefsky, 1977). The excluded fraction was assayed for bound radioactivity, protein content, and ATPase activity. Calculations were based on a M_r of 360000 for F_1.

Quantitative Analysis of Tightly Bound Nucleotides by HPLC. Bound nucleotides were extracted from 50-μg samples of F_1 by heating to 95 °C for 5 min (Tiedge et al. 1982) in TMMg buffer, pH 7.5, in the presence of 2 mM β-mercaptoethanol. The samples were then cooled on ice and centrifuged in a Beckman airfuge. The supernatants were freeze-dried, and the residue was dissolved in 50 μL of water. Nucleotides were analyzed by HPLC. The Waters HPLC apparatus was equipped with a column (250 × 4.6 mm) filled with an anion-exchange phase (Partisil 10-SAX from Whatman) and protected by a guard column (20 × 3 mm) dry-packed with a reverse-phase support (Partisil 10-ODS from Whatman). The UV absorbance at 259 nm of the column effluent was monitored with a Shoeffel 770 spectrophotometer; the peaks were integrated by means of the M730 data module (Waters). Sample injections were controlled with a WISP system (710B) from Waters. The system was calibrated by injecting 200 pmol of ADP or ATP from standard solutions spectrophotometrically titrated. The column was eluted for 15 min with 450 mM KH_2PO_4 at pH 4.5 at a flow rate of 2 mL/min; the peak of ADP appeared after 3.5 min and that of ATP after 11.5 min. Under these conditions, amounts of nucleotides as low as 10–15 pmol could be accurately determined. The peak of 2-azido-ADP appeared at about 4.5 min.

Synthesis of [α-^{32}P]-2-Azido-ADP. (1) Synthesis of 2-Azidoadenosine. Three hundred milligrams of 2-chloroadenosine was incubated for 16 h with 15 mL of anhydrous hydrazine under nitrogen. The mixture was repeatedly evaporated and taken up in water and 1-propanol, and the residue was diazotized (Schaeffer & Thomas, 1958). The crystals of 2-azidoadenosine were washed with cold water on a sintered-glass funnel and dried under vacuum over P_2O_5. The yield from 2-chloroadenosine was about 50%. All subsequent steps were carried out under dim light.

(2) *Synthesis of the 2′,3′-O-Isopropylidene-2-azidoadenosine.* The 2′,3′-isopropylidene acetal derivatives of D-ribonucleosides are useful intermediates in the synthesis of 5′-substituted D-ribonucleosides, especially D-ribonucleoside 5′-phosphates. The 2′,3′-*O*-isopropylidene derivative was prepared as described by Tomasz (1978). One hundred and fifty milligrams of anhydrous 2-azidoadenosine was dissolved in 1.5 mL of anhydrous acetone in the presence of 150 μL of *p*-toluenesulfonic acid dried over P_2O_5 at 60 °C for 1 day. Three hundred and fifty microliters of anhydrous ethyl orthoformate, distilled prior to use and stored over calcium hydride, was added dropwise in 10 min. After 1 h, the mixture was neutralized with ammonium hydroxide. It was treated further with 1 g of charcoal at room temperature and filtered. The yellow filtrate was dried under reduced pressure and redissolved in 2 mL of water; the pH was adjusted to 10 by dropwise addition of 0.1 N NaOH. The 2′,3′-*O*-isopropylidene-2-azidoadenosine was extracted with ethyl acetate. The ethyl acetate was removed by evaporation under reduced pressure, and the resulting gummy residue dissolved in 2 mL of methanol. The purity of 2′,3′-*O*-isopropylideneadenosine (R_f 0.8) was checked by thin-layer chromatography on a silica gel plate (Whatman K6F) developed with petroleum ether–acetic acid–acetone (50:10:20 v/v/v). Its UV spectrum in 10 mM sodium phosphate, pH 7.0, exhibited maxima at λ = 230 and 271 nm and shoulders at 310 and 320 nm. Treatment of 2′,3′-*O*-isopropylideneadenosine with 2 M acetic acid at 90 °C for 45 min removed the protecting isopropylidene group and restored the 2-azidoadenosine, which migrated as a single spot (R_f 0.31).

(3) *Synthesis of* [α-^{32}*P*]*-2-Azido-AMP.* [α-^{32}P]-2-Azido-AMP of high specific radioactivity (10×10^3 dpm/pmol) was synthesized by the method of Symons (1968); it was diluted to the appropriate specific radioactivity with cold 2-azido-AMP prepared from 2′,3′-*O*-isopropylidene-2-azidoadenosine by the method of Sowa & Ouchi (1975), a method more convenient for large-scale preparation.

In the method of Symons (1968), the starting materials were $H_3{}^{32}PO_4$ and 2′,3′-*O*-isopropylidene-2-azidoadenosine; trichloroacetonitrile was used as condensing agent in the presence of triethylamine with dimethyl sulfoxide as solvent. Routinely, a mixture of 1 μmol of $H_3{}^{32}PO_4$ (10 mCi) and 20 μmol of the 2′,3′-*O*-isopropylidene derivative in acetonitrile was dried by three successive evaporations in a rotary evaporator, and the residue was dissolved in 200 μL of anhydrous dimethyl sulfoxide. Twenty micromoles of anhydrous triethylamine and 20 μmol of trichloroacetonitrile distilled over P_2O_5 and stored at −80 °C were added. After incubation at 37 °C for 30 min, the reaction was blocked by 100 μL of ethanol, and the reaction mixture was dried by repeated evaporations of methanol. The dried residue was dissolved in 2 mL of 2 M acetic acid and heated at 90 °C for 45 min. Acetic acid was removed by rotary evaporation; the residue was dissolved in 500 μL of ice-cold 0.1 M acetic acid, and the solution was applied to a 3-mL column of Dowex 50W-X4 H^+ (100–200 mesh) equilibrated with ice-cold 0.1 M acetic acid (Macfarlane et al., 1982). The column was eluted with 4 volumes of 0.1 M ice-cold acetic acid, followed by 8 volumes of ice-cold water. The aqueous fraction containing [α-^{32}P]-2-azido-AMP was concentrated. The purity of [α-^{32}P]-2-azido-AMP was checked by thin-layer chromatography on PEI–cellulose F in 2 M sodium formate, pH 3.6. The product migrated as a single spot (R_f 0.5) (Figure 1). The yield, estimated by UV absorbance, was about 40% with respect to the added [^{32}P]P_i; calculation was based on the assumption that in 0.1 M HCl

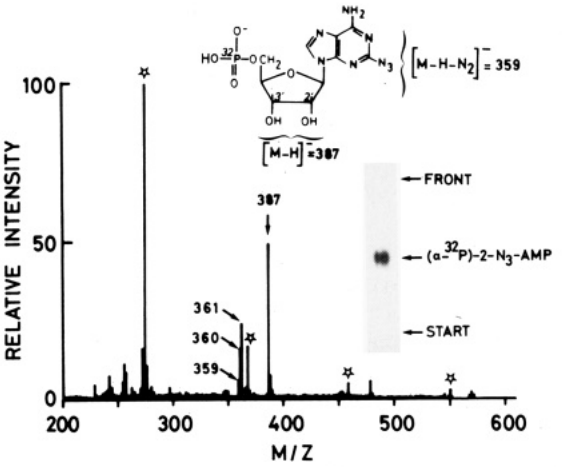

FIGURE 1: Fast atom bombardment mass spectrum of the 2-azido-AMP, showing the quasi-molecular ion $(M - H)^-$ at m/z 387. Peaks with a star correspond to glycerol and its oligomers. (Insert) Ascending chromatography on PEI–cellulose F in 2 M sodium formate, pH 3.6, showing the radiochemical purity of [α-^{32}P]-2-azido-AMP.

the product is entirely in the azido form, with a molar extinction coefficient of 15 500 at 271 nm. Fast atom bombardment mass spectroscopy revealed the presence of the anion $(M - H)^-$ at m/z 387 and three fragments that corresponded to elimination of a nitrogen molecule $(M - N_2 - H)^-$ at m/z 359, followed by the production in the glycerol matrix of hydrogenation products (peaks at m/z 360 and 361). The peaks at m/z 275, 368, 459, and 551 were due to deprotonated glycerol oligomers (Figure 1). The newly synthesized [α-^{32}P]-2-azido-AMP was diluted with the unlabeled derivative to a specific radioactivity of about 0.4–0.5 Ci/mmol.

Nonradioactive 2-azido-AMP was prepared on a larger scale by the method of Sowa & Ouchi (1975) by adding to the 2′,3′-*O*-isopropylidene-2-azidoadenosine (40 μmol) in 1 mL of anhydrous acetonitrile 2 mL of the following mixture: $POCl_3$–H_2O–pyridine (1/0.5/1 mmol/mmol/mmol, in 5 mL of CH_3CN) freshly prepared at 0 °C. After 4 h in the dark at 0 °C, the reaction was quenched with 1 mL of cold water, and the mixture was left overnight with stirring. After evaporation of solvents, the dried residue was redissolved in 2 mL of 2 N acetic acid, and the solution was heated at 90 °C for 45 min to remove the isopropylidene group. The unlabeled 2-azido-AMP was purified following the same method as that used for [α-^{32}P]-2-azido-AMP. The yield of recovery was on average 40% with regard to 2′,3′-*O*-isoproylidene-2-azidoadenosine. Both labeled and unlabeled 2-azido-AMP exhibited the same chromatographic behavior, the same mass spectrum, and the same UV spectrum.

(4) *Phosphorylation of* [α-^{32}*P*]*-2-Azido-AMP into* [α-^{32}*P*]*-2-Azido-ADP.* [α-^{32}P]-2-Azido-ADP was prepared by the method of Hoard & Ott (1965) with [α-^{32}P]-2-azido-AMP (3 μmol) activated by *N*,*N*′-carbonyldiimidazole (20 μmol) and the tri-*n*-butylammonium salt of orthophosphate (20 μmol). The reaction mixture was then dried and redissolved in 0.5 mL of acetic acid, 0.1 M at 0 °C. [α-^{32}P]-2-Azido-ADP was purifed from the nonphosphorylated [α-^{32}P]-2-azido-AMP by passage through a 2-mL bed column of Dowex 50W-X4 H^+ (100–200 mesh) equilibrated in 0.1 M ice-cold acetic acid. The pass-through fraction (4 column volumes of 0.1 M ice-cold acetic acid) was concentrated and filtered through a Sep-Pak C_{18} cartridge (Waters) washed with 5 mL of water. The eluted fraction contained P_i and [α-^{32}P]-2-azido-ADP. It was concentrated by rotary evaporation, and the purity of [α-^{32}P]-2-azido-ADP was assessed by its migration as a single radioactive spot in the following systems of chromatography: (1) PEI–

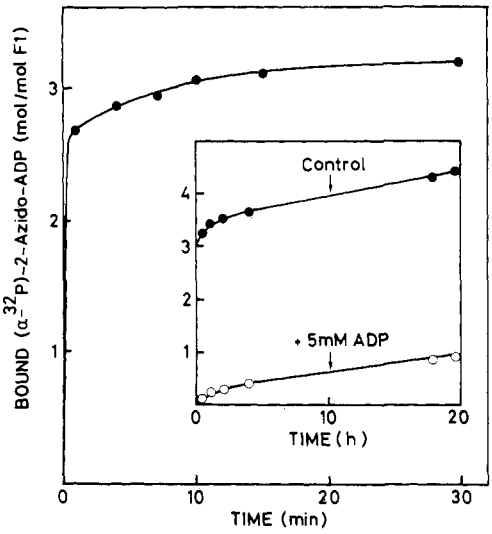

FIGURE 2: Time dependence of the reversible binding of [α-^{32}P]-2-azido-ADP to mitochondrial F_1 in the dark. F_1 was incubated at 1 mg/mL with 140 μM [α-^{32}P]-2-azido-ADP in TMMg buffer in the dark (at 20 °C), in a total volume of 1.5 mL. At the time indicated, 100-μL aliquots were transferred to small centrifuge columns filled with Sephadex G-50 (fine). Column effluents were collected for protein determination and radioactivity counting. (Insert) Effect of ADP on the binding of [α-^{3P}]-2-azido-ADP in the dark; 5 mM ADP was added after incubation with [α-^{32}P]-2-azido-ADP; after 15 min, the samples were processed by centrifugation–filtration.

cellulose F in 2 M sodium formate, pH 3.6 ($R_f \simeq 0.2$); (2) cellulose F in 1-propanol–11 N NH_4OH–H_2O (55:20:25 v/v/v) (R_f 0.4); (3) silica gel 60F in dioxane–2-propanol–11 N NH_4OH–H_2O (40:20:36:34 v/v/v/v) (R_f 0.6). Fast atom bombardment mass spectroscopy in the negative ion mode revealed the quasi-molecular ion $(M - H)^-$ at m/z 467. The yield was determined by UV absorbance of an aliquot sample in 0.1 M HCl; the calculation was based on the same molar extinction coefficient as used for 2-azido-AMP. The product was dissolved in 2% ethanol in its nonneutralized form (pH 4–5). It has recently been recalled that 2-azido-ADP can be kept in its azido form only at acidic pH (0.1 N HCl). In fact, at neutral pH, 2-azido-ADP slowly isomerizes into tetrazolo derivatives, with an equilibrium constant of about 1 (Czarnecki, 1984). Since only the 2-azido-ADP is photoactivable, in the experiments described thereafter the mixture of 2-azido-ADP and the tetrazolo isomers will be referred as 2-azido-ADP.

RESULTS

Reversible Binding Parameters of [α-^{32}P]-2-Azido-ADP in the Dark. The reversible binding of [α-^{32}P]-2-azido-ADP to F_1 in the dark was assayed with a saturating concentration of the probe (140 μM) (see below). In a Mg^{2+}-supplemented medium at 20 °C, kinetics were typically biphasic. The rapid phase lasted for less than 1 min and corresponded to the binding of 2.8 mol of [α-^{32}P]-2-azido-ADP/mol of F_1; after 1 min of incubation, the amount of bound [α-^{32}P]-2-azido-ADP/mol of F_1 slowly increased, amounting to 4.3 mol of bound probe/mol of F_1 after 20 h of incubation (Figure 2).

The binding affinity and specificity were assayed by incubating F_1 with increasing concentrations of [α-^{32}P]-2-azido-ADP for 10 min at 20 °C in the dark (Figure 3). This incubation period corresponds to the rapid phase of binding. Three moles of [α-^{32}P]-2-azido-ADP/mol of F_1 was found to bind at saturation with a K_d value of 5 μM in a Mg^{2+}-supplemented medium. In the presence of EDTA, the amount of bound [α-^{32}P]-2-azido-ADP leveled up to 2 mol/mol of F_1, and the K_d value was 30 μM. The bound radioactivity corresponding to the rapid phase of binding was removed by addition of a large excess (5 mM) of unlabeled ADP. In contrast, the slowly bound 2-azido-ADP, which accumulated after a few hours, was not displaced by ADP (Figure 2), suggesting that it may substitute for the tightly bound nucleotides.

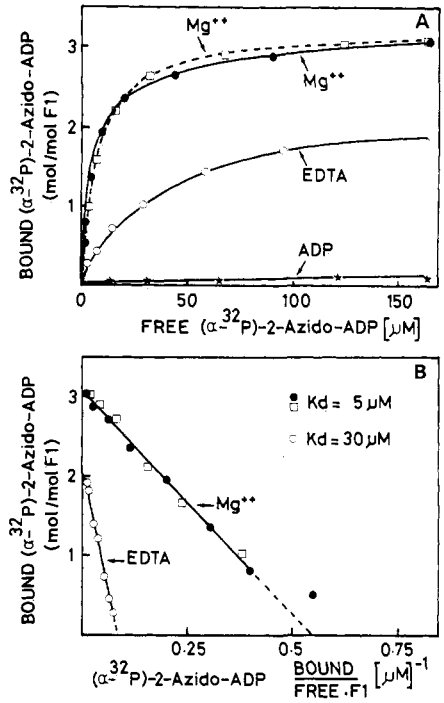

FIGURE 3: Reversible binding of [α-^{32}P]-2-azido-ADP and [α-^{32}P]-tetrazolo-ADP to mitochondrial F_1 in the dark. (A) F_1 (100 μL, 1 μM) was incubated in TMMg or TMES buffer with [α-^{32}P]-2-azido-ADP with or without 5 mM ADP in the dark for 10 min at 20 °C. The tetrazolo forms were obtained by incubation in 0.1 N NaOH for 4–5 h; the mixture was neutralized and incubated for 5 min with F_1. The final concentrations of added α-^{32}P-labeled nucleotide varied from 1.5 to 170 μM. After incubation, the unbound ligand was removed by centrifugation–filtration as described under Materials and Methods. The binding stoichiometries were determined on the eluates. (—) α-^{32}P-Labeled nucleotide as an equimolar mixture of azido and tetrazolo forms in the presence of Mg^{2+} (●), in the presence of EDTA (○), or in the presence of 5 mM ADP (★); (– –) α-^{32}P-Labeled tetrazolo-ADP in the presence of Mg^{2+} (□). (B) Scatchard plot of the binding data.

In the experiment corresponding to Table I, mitochondrial F_1 was incubated with [α-^{32}P]-2-azido-ADP for different periods of time resulting in an increased amount of bound photolabel. Binding of [α-^{32}P]-2-azido-ADP was accompanied by the release of tightly bound ADP and ATP. A short period of incubation (0.5 h) of [α-^{32}P]-2-azido-ADP with F_1 resulted in the binding of 3.1 mol of photolabel/mol of F_1; 2.3 ± 0.3 mol of endogeneous nucleotides/mol of F_1 was still present compared to 3.5 mol/mol of F_1 prior to binding of the photolabel. Upon longer periods of incubation, more photolabel bound to F_1, and concomitantly, a similar amount of endogeneous nucleotides was released. In the presence of [α-^{32}P]-2-azido-ADP, whatever the length of the period of incubation, the total amount of endogenous nucleotides and bound photolabel never exceeded 6 mol/mol of F_1.

It should now be recalled that, at neutral pH, 2-azido-ADP is in equilibrium with tetrazolo isomers, the equilibrium constant being about 1 (Czarnecki, 1984). In all preceding experiments, the concentration of free ligand was calculated in first approximation by assuming that both species, the 2-azido-ADP and its tetrazolo isomers, had the same binding affinity for F_1. This hypothesis could be tested readily since

Table I: ADP and ATP Content of F_1-ATPase as a Function of the Binding of [α-^{32}P]-2-Azido-ADP at Saturating Concentration in the Dark

incubation period (h)[a]	[α-^{32}P]-2-azido-ADP (μM)	tightly bound ADP and ATP (mol/mol of F_1)	bound [α-^{32}P]-2-azido-ADP (mol/mol of F_1)	total amount of bound nucleotides (mean) (mol/mol of F_1)
0 (a)	none	2.9 ± 0.2	none	2.9
0 (b)	none	3.5 ± 0.2	none	3.5
0.5 (b)	140	2.3 ± 0.3	3.1 ± 0.1	5.4
2 (b)	140	2.1 ± 0.3	3.4 ± 0.1	5.5
4 (b)	140	1.8 ± 0.3	3.8 ± 0.1	5.6
20 (b)	140	1.2 ± 0.2	4.3 ± 0.1	5.5
20 (b)	none	3.4 ± 0.3	none	3.4

[a] F_1-ATPase, stored as a precipitate in $(NH_4)_2SO_4$ in the presence of 4 mM ATP, was desalted on a Sephadex G-50 column equilibrated in 0.25 M sucrose–50 mM Tris–acetate, pH 7.5. The F_1-bound ADP and ATP were determined as follows: (a) desalted F_1 precipitated by $(NH_4)_2SO_4$ and filtered on Sephadex G-50 in TMMg buffer or (b) desalted F_1 treated or not by the 2-azido-ADP in the dark followed by filtration of Sephadex G-50. For [α-^{32}P]-2-azido-ADP binding, the desalted F_1 was incubated for different periods of time in the dark with 140 μM [α-^{32}P]-2-azido-ADP in 50 mM Tris, 50 mM MES, 50 mM sucrose, and 2 mM $MgCl_2$, pH 7.5. The bound nucleotides, ADP, ATP, and [α-^{32}P]-2-azido-ADP, were determined on column eluates. Nucleotides were extracted from F_1 by heat treatment and analyzed by HPLC (cf. Materials and Methods). Results are the mean of determinations in three to five experiments ± SD.

it is possible to transform the 2-azido nucleotides into tetrazolo isomers almost completely, by treatment at alkaline pH (0.1 N NaOH) at 20 °C; upon neutralization, the return to equilibrium is very slow, taking about 0.5 h for completion (Czarnecki, 1984). Because binding is a fast reaction (Figure 2), advantage was taken of the slow back-formation of 2-azido-ADP from the tetrazolo isomers. The α-^{32}P-labeled tetrazolo isomers were incubated with F_1 for 5 min, and the F_1-bound radioactivity was assessed after gel filtration. Although some azido-ADP accumulated during the course of this incubation, the major part of the derivatives still consisted of the tetrazolo isomers. The binding curve obtained under these conditions was virtually superimposable on the curve obtained with a mixture in equal concentrations of 2-azido-ADP and the tetrazolo derivatives (Figure 3), indicating that in the dark 2-azido-ADP and the derived isomers have a similar affinity for mitochondrial F_1.

Photoaffinity Labeling Experiments. (1) Correlation between Photolabeling of F_1 and ATPase Inactivation. Preincubation of F_1 with [α-^{32}P]-2-azido-ADP in the dark for 10 min at 20 °C, followed by photoirradiation for 3 s, resulted in inactivation of the ATPase activity and covalent incorporation of radioactivity into F_1. Under similar conditions of irradiation in the absence of 2-azido-ADP, there was no loss of ATPase activity in F_1. The extent of photolabeling was varied by modifying the concentration of [α-^{32}P]-2-azido-ADP (from 1 to 20 μM) in a Mg^{2+}-supplemented medium. To remove the noncovalently bound azido-ADP and photoproducts, 5 mM ADP was added to the photolabeled F_1 and left in contact for 15 min before filtration on Sephadex G-50. A linear relationship between the bound [α-^{32}P]-2-azido-ADP and the loss of activity was found up to 40–50% inactivation (Figure 4A). Above 50% inactivation, the plots became curvilinear, as if more bound azido-ADP was required for inactivation. The same curvilinear plots were obtained in a medium supplemented with $MgCl_2$ or EDTA. The curvilinearity of the plots could result from lack of efficiency of ADP to induce complete release of noncovalently bound azido-ADP or derived photoproducts. Heat treatment was therefore attempted. The precipitated F_1 was sedimented by centrifugation in a Beckman airfuge, and the bound radioactivity in the pellet was counted. A linear relationship between inactivation and photolabeling was now observed up to 90% inactivation. By extrapolation, it was found that 100% inactivation corresponded to 2 mol of azido derivatives bound per mole of F_1 (Figure 4B).

The inhibition resulting from photolabeling was purely noncompetitive; i.e., the fraction of F_1 that remained nonlabeled and was still active had the same affinity for ATP as

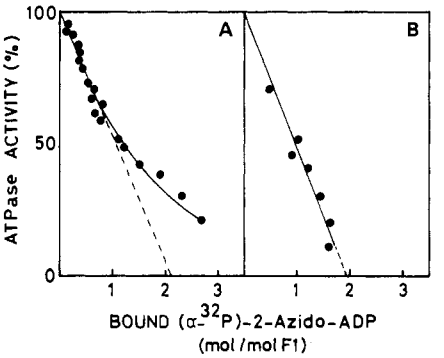

FIGURE 4: Correlation between photolabeling of mitochondrial F_1 with [α-^{32}P]-2-azido-ADP and ATPase inactivation. (A) After 10 min of preincubation in the dark, mitochondrial F_1 (100 μg) was photoirradiated for 3 s at 20 °C with concentrations of [α-^{32}P]-2-azido-ADP ranging from 1 to 20 μM in the TMMg buffer. The photolabeled F_1 samples were then incubated with 5 mM ADP for 15 min. The unreacted [α-^{32}P]-2-azido-ADP and photoproducts were eliminated by centrifugation–filtration. The ATPase activity, the bound radioactivity, and the protein content were determined on column eluates as described under Materials and Methods. (B) Same experiment as in Figure 4A, except that, after photolabeling with [α-^{32}P]-2-azido-ADP and treatment with 5 mM ADP, a 50-μg aliquot of photolabeled F_1 was heated at 95 °C for 5 min. The precipitated F_1 was recovered by centrifugation in a Beckman airfuge. The bound radioactivity was determined on the F_1 precipitate.

the native F_1 ($K_M \simeq 140$ μM). The photolabeling specificity of [α-^{32}P]-2-azido-ADP was corroborated by the protective effect of an excess ADP, added prior to photoirradiation, on F_1 inactivation.

(2) Effect of Preincubation Conditions on Photolabeling. In a preliminary paper, it was reported that the β subunit of mitochondrial F_1 is specifically photolabeled by [α-^{32}P]-2-azido-ADP after a 1-min preincubation of F_1 with the photolabel (Dalbon et al., 1985). Due to the fact that 2-azido-ADP carries three negative charges at neutral pH, the photolabeled β subunits could be easily differentiated from the unlabeled β subunits by electrophoretic migration (Figure 5). Advantage was taken of this differential migration to answer the question of whether the two azido-ADP that are required to inactivate F_1 bind to two sites on the same β subunit or to two different β subunits, each of which carries only one site. Assuming two sites on the same subunit, inactivation of one β subunit out of the three present in F_1 should lead to full inactivation; consequently, one-sixth of the β subunits in the F_1 population would be expected to be modified at half-inactivation. In the second alternative (binding of two β subunits), one-third of the β subunits in the F_1 population would be modified at half-inactivation. The labeling data in Figure

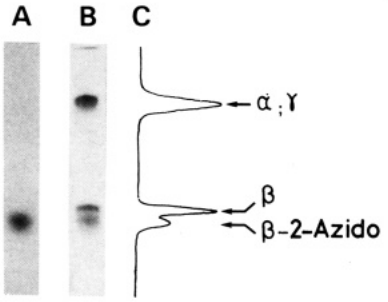

FIGURE 5: Electrophoretic separation of photolabeled and unlabeled β subunits after photolabeling of mitochondrial F_1 with [α-^{32}P]-2-azido-ADP. F_1 was photoirradiated for 3 s with 20 μM [α-^{32}P]-2-azido-ADP after 10 min of preincubation in the dark. Photoirradiation resulted in about 50% inactivation of F_1, corresponding to the covalent binding of 1 mol of photolabel/mol of F_1. The photoirradiated sample was freeze-dried, redissolved in 8 M urea, subjected to urea–polyacrylamide gel electrophoresis at pH 8.5, and stained with Coomassie Blue as described under Materials and Methods. After drying, the gel was exposed to a Fuji RX film for 24 h. (A) Fluorography; (B) Coomassie Blue staining; (C) densitometric trace of the gel stained by Coomassie Blue. The percentage of photolabeled and unlabeled β subunits was calculated from the areas of the densitometric trace.

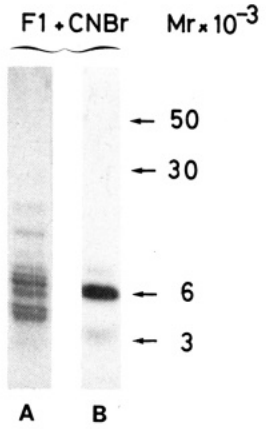

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of cyanogen bromide peptides obtained from photolabeled mitochondrial F_1. In this experiment, the stoichiometry of the photolabeling was 1.5 mol of bound [α-^{32}P]-2-azido-ADP/mol of F_1. The enzyme was treated overnight with cyanogen bromide in 70% formic acid (cf. Materials and Methods). The cyanogen bromide peptides were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis and stained by Coomassie Blue (track A); the ^{32}P-labeled peptides were revealed by fluorography (track B).

5 correspond to about 50% inactivation of F_1 by [α-^{32}P]-2-azido-ADP; only the β subunits were photolabeled. The distribution pattern of the β subunits after gel electrophoresis indicated that about one-third of the β subunits was modified by photolabeling, in agreement with the second alternative. One can therefore conclude that one azido-ADP binds to one β subunit and that the modification of two β subunits per F_1 results in full inactivation. Consistent with this conclusion is the finding that [α-^{32}P]-2-azido-ADP binds covalently to a discrete region of the β subunit; in fact, after cyanogen bromide cleavage of photolabeled F_1, a major radioactive band, with a molecular weight of about 6000, was revealed among numerous bands stained by Coomassie blue, which corresponded to cleaved peptides (Figure 6).

In the above experiments, photolabeling was performed after 10 min of preincubation in the dark. Under these conditions, the bound [α-^{32}P]-2-azido-ADP was fully removed by ADP, and the subsequent photoirradiation led to exclusive binding of the β subunit. When preincubation in the dark was performed for several hours, extra binding of [α-^{32}P]-2-azido-ADP occurred, which was not reversed by ADP; yet, ADP displaced

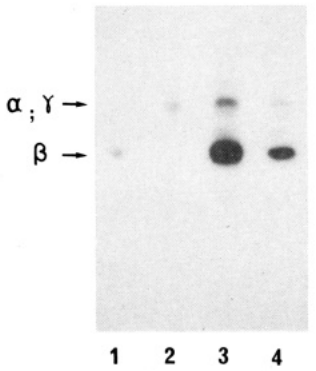

FIGURE 7: Covalent photolabeling of mitochondrial F_1 by [α-^{32}P]-2-azido-ADP after a long-term preincubation in the dark. F_1-ATPase (1 mg/mL) was incubated for 20 h with [α-^{32}P]-2-azido-ADP at the saturating concentration of 140 μM in 50 mM Tris, 50 mM MES, 50 mM sucrose, and 2 mM $MgCl_2$, pH 7.5. Incubation was followed by filtration of an aliquot sample on a Sephadex G-50 column. A second aliquot was incubated with 5 mM ADP for 15 min before filtration on a Sephadex G-50 column. The column eluates were photoirradiated for 3 s at 20 °C. The photoirradiated samples were freeze-dried and redissolved in 8 M urea. Five-microgram aliquots were subjected to urea–polyacrylamide gel electrophoresis (Knowles & Penefsky, 1972), followed by autoradiography as described under Materials and Methods. Track 1 corresponds to the migration of subunit β of F_1 labeled by [^{14}C]DCCD (Pougeois et al., 1979). Track 2 corresponds to α and γ subunits labeled by [^{14}C]NEM (Senior, 1975). Tracks 3 and 4 correspond to F_1 photoirradiated in the presence of [α-^{32}P]-2-azido-ADP prior to and after treatment with 5 mM ADP, respectively.

the 3 loosely bound mol of photolabel. The extra bound, nondisplaceable [α-^{32}P]-2-azido-ADP behaved as a tightly bound nucleotide. When the long-term preincubation in the dark was followed by Sephadex G-50 filtration and F_1 was photoirradiated, the β subunit became extensively labeled and the α subunit was labeled to some extent; α contained about 10% of the total bound radioactivity (Figure 7, track 3). In the experiment illustrated by Figure 7, the β subunit was separated from the block of the α and γ subunits by urea–polyacrylamide gel electrophoresis. The labeling of the α subunits was ascertained by a further electrophoretic migration in $NaDodSO_4$–polyacrylamide gel (not shown). When the long-term preincubation in the dark was followed by addition of 5 mM ADP, prior to gel filtration, a substantial decrease of covalently bound radioactivity was observed both in the α and β subunits; only traces of radioactivity remained in the α subunit (Figure 7, track 4). Photolabeling of the α subunit depended therefore on whether the long-term preincubation of F_1 with [α-^{32}P]-2-azido-ADP in the dark was followed by treatment with ADP prior to gel filtration and photoirradiation.

An interesting question raised at this point was that of the location of the extra bound [α-^{32}P]-2-azido-ADP on the β subunit. By limited enzymatic proteolysis of photolabeled F_1 with *S. aureus* V8 protease, different patterns of distribution of radioactive bands corresponding to labeled peptides were obtained, depending on whether F_1 was photolabeled with [α-^{32}P]-2-azido-ADP in the dark either for a short period or for a long period followed by treatment with ADP (Figure 8). In the latter case, the loosely bound [α-^{32}P]-2-azido-ADP corresponding to the initial period of preincubation was released by ADP (see Figure 2). The two different patterns of photolabeling obtained make it clear that there are two binding sites for [α-^{32}P]-2-azido-ADP on the β subunit. One of these sites is filled rapidly by the photolabel in the dark and is also rapidly unloaded upon addition of ADP; this site is probably catalytic and accounts for the two-thirds of the site reactivity of F_1 with respect to 2-azido-ADP (Figure 4). The second

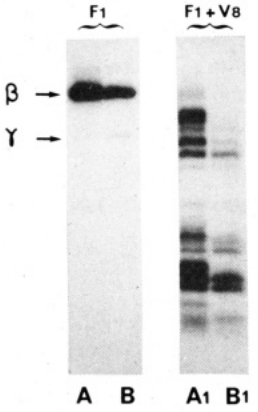

FIGURE 8: Partial proteolytic digestion of mitochondrial F_1 photolabeled after short and long preincubation in the dark with [α-^{32}P]-2-azido-ADP. F_1 was incubated in the dark with 140 μM [α-^{32}P]-2-azido-ADP as described in Figure 7. After 10 min, an aliquot was withdrawn, desalted through a Sephadex G-50 column, and irradiated for 3 s (A). After 20 h of incubation, a second aliquot was withdrawn and left in contact with 5 mM ADP for 15 min before desalting by passage through a Sephadex G-50 column. The column eluate was photoirradiated for 3 s (B). Fifty-microgram aliquots of samples A and B were digested with 5 μg of *S. aureus* V8 protease for 1 h in 50 mM ammonium bicarbonate and freeze-dried. The respective digests, A_1 and B_1, together with the original samples A and B were subjected to $NaDodSO_4$ slab gel electrophoresis followed by autoradiography as described under Materials and Methods.

site of the β subunit is slowly filled with [α-^{32}P]-2-azido-ADP; the photolabel, once bound in the dark, is not released readily upon addition of ADP.

Discussion

Advantages and Drawbacks of [α-^{32}P]-2-Azido-ADP as a Photolabel of F_1. The synthesis of [β-^{32}P]-2-Azido-ADP was previously described by MacFarlane et al. (1982) and Czarnecki et al. (1982). The chemical synthesis of 2-azido-ADP with [^{32}P]P_i in the α-position described here was more tedious. However, unlike [β-^{32}P]-2-azido-ADP, [α-^{32}P]-2-azido-ADP did not lose its radioactivity when left in contact with acid reagents, such as 70% formic acid, which is commonly used in peptide mapping studies. The stability of the [α-^{32}P]-2-azido-ADP is an obvious advantage for mapping and stoichiometric studies.

Other photoactivable nucleotide derivatives that have been used to explore the nucleotide binding sites of F_1 include the 8-azidopurine derivatives, the 3′-ribose-coupled arylazido derivatives of ADP, ATP, and AMPPNP, the 3′-ribose-coupled azidonaphthoyl derivative of ADP, and the 3′-ribose-coupled benzoylbenzoyl derivative of ATP (see the introduction). A number of the above derivatives have drawbacks inherent in their structure. For example, the substitution at the purine C-8 position represents a small structural change, but it shifts the nucleotide conformation about the N-glycosidic linkage from anti to syn (Sarma et al., 1974), which may explain the low affinity of 8-azido-ATP for F_1 (Schäfer et al., 1976). Some of the 3′-ribose derivatives in which the azido group is separated from the ribose moiety by a long arm may cause difficulty in the interpretation of the labeling data (Lunardi et al., 1982; Williams & Coleman, 1982; Lübben et al., 1984). The 2-azido-ADP (ATP) seems to retain the anti conformation (Czarnecki et al., 1982), which is characteristic of natural ADP (ATP) (Haschemeyer & Rich, 1967). An apparent drawback of the 2-azido nucleotides is, however, their tautomerization into tetrazole derivatives.

The azido–tetrazolo equilibrium depends on pH. For example, the azido isomer predominates at acidic pH, and the tetrazolo forms at alkaline pH. The return to equilibrium is slow; in 5 min less than 10% of the return to equilibrium is attained at 20 °C. Because binding is much more rapid than return to equilibrium, it has been possible by manipulating the tautomeric equilibrium to show that the tetrazolo isomers in the dark are able to bind to the nucleotide binding sites of F_1 with the same affinity as the azido isomer. The presence of nonphotoactivable tetrazolo isomers beside 2-azido-ADP might explain the departure from linearity of the correlation curve between photolabeling and inactivation above 50% inactivation in the absence of heat treatment of the photolabeled F_1. It should be recalled that, prior to heat treatment, the photolabeled F_1 was treated with an excess of ADP and passed through a Sephadex column. Strict linearity in the correlation curve was obtained after heating the photolabeled enzyme and assaying the bound radioactivity in the precipitated enzyme. Most likely, heat treatment eliminated entrapped, but noncovalently bound tetrazolo isomers.

Dependence of Photolabeling on Preincubation Conditions in the Dark. At least three different conditions of preincubation of mitochondrial F_1 in the dark with [α-^{32}P]-2-azido-ADP can be delineated, which lead to different results with respect to the extent and the topography of photolabeling. (1) When F_1 was preincubated in the dark with [α-^{32}P]-2-azido-ADP for short periods of time (e.g., 10 min), the bound radioactivity was totally displaced by excess ADP and binding was specifically directed toward the β subunit, as shown by subsequent photolabeling. Under these conditions, up to 3 mol of photolabel/mol of F_1 could bind to the enzyme in the presence of $MgCl_2$. (2) When preincubation of F_1 with [α-^{32}P]-2-azido-ADP in the dark was conducted for longer periods of time (up to 20 h) and at saturating concentrations of the photolabel, up to 4.3 mol of photolabel/mol of F_1 was found to bind; photoirradiation after Sephadex G-50 filtration led to photolabeling of both the α and β subunits, with the β subunit being labeled about 10 times more than the α subunit (Figure 7, track 3). (3) When the long-term preincubation in the dark was followed by addition of excess ADP before photoirradiation, the extent of photolabeling was decreased and the photolabel was attached mainly to the β subunit. Taken together, these data indicate that upon photoirradiation the extra bound [α-^{32}P]-2-azido-ADP (after long-term preincubation) binds covalently to tight sites in both the α and β subunits, with marked preference for the β subunit. These tight sites are thought to be filled with the endogenous tightly bound ADP or ATP in the native enzyme. They are clearly different from the loose sites labeled by [α-^{32}P]-2-azido-ADP after short-term preincubation, as demonstrated by mapping studies after partial digestion of F_1 with *S. aureus* V8 protease (Figure 8). In other words, the β subunit would contain two binding sites, one of which is filled with loosely bound ADP and ATP in the native enzyme and is capable of reacting rapidly with [α-^{32}P]-2-azido-ADP while the other is a tight binding site that is filled slowly with [α-^{32}P]-2-azido-ADP and is shared with the α subunit. In summary, the binding sites for the extra bound 2-azido-ADP (tight sites) would be located at the junction of the α and β subunits; the fact that the photolabeling of α was more limited than that of β could be due to the orientation of the probe. These data are compatible with six nucleotide binding sites per F_1, three loose sites belonging to the β subunits and three tight sites being shared by the α and β subunits.

Partial Site Reactivity of Mitochondrial F_1. The β subunit of mitochondrial F_1 is believed to contain the catalytic site of the enzyme. The correlation of the photolabeling of two β subunits out of the three present in F_1 with full inactivation

of F_1 after short-term preincubation with [α-^{32}P]-2-azido-ADP recalls the partial site reactivity of F_1 obtained with 8-azido-ATP in the presence of EDTA (Wagenvoord et al., 1977). However, in photolabeling by a number of ADP or ATP derivatives including 8-azido-ADP or -ATP in the presence of Mg^{2+} (Wagenvoord et al., 1979), 3'-(arylazido)-ADP (Lunardi et al., 1977; Weber et al., 1985), (benzoylbenzoyl)-ATP (Williams & Coleman, 1982), 3'-(arylazido)-AMPPNP (Lunardi & Vignais, 1982), (azidonaphthoyl)-ADP (Lübben et al., 1984), both α and β subunits were labeled. When both α and β subunits were labeled, full inactivation was achieved, irrespective of the proportion of each labeled subunit, indicating that although the α subunit does not contain the catalytic site of F_1, its function in catalysis is critical enough for its chemical modification to lead inactivation. Labeling of both α and β subunits by azido nucleotides has been taken as evidence that α and β subunits combine to form the proper configuration of the catalytic center (Lübben et al., 1984). However, this is difficult to reconcile with the fact that F_1 with two photolabeled β subunits and no photolabeled α subunit was fully inactivated. It must be stressed that photolabeling of two β subunits leading to inactivation occurred under conditions of preincubation where only the rapidly exchangeable binding sites (probably catalytic sites) were loaded with [α-^{32}P]-2-azido-ADP.

The stoichiometry of inactivation corresponding to a two-thirds of the site reactivity is puzzling per se. In fact, in the well-accepted alternate site mechanism (Boyer et al., 1977), with three β subunits acting alternatively as active catalysts, it is easy to conceive that the covalent modification of one β subunit results in full inactivation of the whole F_1. Photolabeling of two β subunits in a molecule of F_1 is expected to occur sequentially. In fact, if each of these two β subunits were labeled at the same rate, there would be no reason for a molecule of [α-^{32}P]-2-azido-ADP to react more readily with a molecule of F_1 already labeled on one of its β subunit (and inactivated) than with an unlabeled molecule of F_1. This statistical distribution would result in a correlation curve not obligatorily linear, extrapolating to a value of less than 2 mol of bound azido-ADP for 100% inactivation. This is at variance with the data in Figure 4. The alternative hypothesis is to consider that, following the binding of a first molecule of [α-^{32}P]-2-azido-ADP to one of the three subunits of F_1, a change of conformation occurs immediately, which makes a second β subunit highly reactive to the azido derivative. In other words, the two labeling reactions would proceed quasi-concomitantly. Whether inactivation of F_1 is associated with the labeling of the first β subunit or of the second is undecided. It is even possible that the conformational change that is supposed to follow the labeling of the first β subunit and to preceed the labeling of the second plays a decisive role in F_1 inactivation.

Acknowledgments

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Registry No. ATPase, 9000-83-3; 2-azido-ADP, 64020-53-7; ADP, 58-64-0; 5'-ATP, 56-65-5; tetrazolo-ADP ([5,1-*b*] isomer), 80134-53-8; tetrazolo-ADP ([5,1-*a*] isomer), 80134-52-7; Mg, 7439-95-4.

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