Photolabeling of Mitochondrial F_1-H^+ATPase by 2-Azido[^{3}H]ADP and 8-Azido[^{3}H]ADP Entrapped as Fluorometal Complexes into the Catalytic Sites of the Enzyme†

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ABSTRACT: In the presence of ADP and fluorometals, the ATPase activity of the catalytic sector, F_1, of beef heart mitochondrial ATPase is strongly inhibited; this inhibition is dependent on the entrapment of ADP–fluoroaluminate complexes into the nucleotide binding sites of F_1 [Lunardi, J., Dupuis, A., Garin, J., Issartel, J. P., Michel, L., Chabre, M., & Vignais, P. V. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 8958–8962]. We describe here the effect of fluoroaluminate on the binding of 2-azido[^{3}H]ADP and 8-azido-[^{3}H]ADP to beef heart mitochondrial F_1 in the absence and presence of light. When the incubation medium was supplemented with NaF and $AlCl_3$, and maintained in the dark, both 2-azido[^{3}H]ADP and 8-azido-[^{3}H]ADP were able to elicit inhibition of F_1-ATPase activity, exactly like ADP did. Upon photoirradiation, 2-azido[^{3}H]ADP and 8-azido[^{3}H]ADP bound covalently to F_1. Labeling was restricted to the β subunit of F_1, and the same tyrosine residue, β-Tyr-345, was labeled by either of the photoprobes. This is in contrast with the previous findings that in the absence of fluoroaluminate both the α and β subunits of F_1 were photolabeled by 8-azido[^{3}H]ADP, and that two different regions of the β subunit were labeled, centered on β-Tyr-345 in the case of 2-azido[^{3}H]ADP [Garin, J., Boulay, F., Issartel, J. P., Lunardi, J., & Vignais, P. V. (1986) *Biochemistry 25,* 4431–4437] and β-Tyr-311 in that of 8-azido[^{3}H]ADP [Hollemans, M., Runswick, M., Fearnley, I. H., & Walker, J. E. (1983) *J. Biol. Chem. 258,* 9307–9313]. It is postulated that fluoroaluminate nucleotide complexes promote a rearrangement of the architecture of the catalytic site of F_1 that enables the two opposite sides of the adenine ring of the bound nucleotide to interact with the same peptide segment of the β subunit.

The catalytic sector of the ATP synthase complex, F_1,[1] is composed of five different subunits with a well-defined stoichiometry, $\alpha_3\beta_3\gamma\delta\epsilon$. The major subunits, α and β, contain the ADP/ATP binding sites. Out of the six F_1 nucleotide binding sites, only three rapidly exchange their bound nucleotide under conditions of ATP hydrolysis; they are referred to as "exchangeable sites" and are considered as potential catalytic sites [for a review, see Issartel et al. (1992)]. Mapping of the nucleotide binding sites of F_1 has benefited from the use of a number of nucleotide analogues, namely, 8-azido-ATP (Hollemans et al., 1983), 2-azido-ADP (Garin et al., 1986; Xue et al., 1987), azidonitrophenyl phosphate (Garin et al., 1989), FSBI (Bullough & Allison, 1986), and FDNP–ADP (Chuan & Wang, 1988). On the basis of the results of labeling experiments and mutagenesis studies, models of the structural organization of the F_1 nucleotide binding sites have been proposed (Garin et al., 1986, 1989; Ida et al., 1991; Horbach et al., 1991; Vogel & Cross, 1991; Zhuo et al., 1992; Harris, 1993; Iwamoto et al., 1993; Weber et al., 1993). A further step in the understanding of the mechanism of F_1-ATPase has been the study of inhibition of F_1 by fluoride and aluminum ions (Lunardi et al., 1988). It was shown that treatment of F_1 by fluorometals in the presence of ADP results in the very tight binding of ADP to F_1 and in strong inhibition of the enzyme. It was postulated that the ADP–fluoroaluminate complex binds to the catalytic sites of F_1 which adopt a conformation close to that exhibited in the transition state during the normal course of catalysis (Dupuis et al., 1989). It was therefore tempting to extend earlier studies on photolabeling of mitochondrial F_1 with 2-azido-ADP (Garin et al., 1986) and 8-azido-ATP (Hollemans et al., 1983), and to find out whether the entrapment of the azido-nucleotides into the active site of the enzyme upon addition of fluoroaluminate would modify the nature of the photolabeled amino acid residues. We indeed demonstrate that the presence of fluoroaluminate promotes drastic conformational changes in the catalytic crevice of the enzyme.

MATERIALS AND METHODS

Chemicals. 2-Chloroadenosine was from Sigma and 2-chloro[8-^{3}H]adenosine from Moravek (Moravek Biochemicals, Inc.). AMP was purchased from Boehringer, and [2-^{3}H]-

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[1] Abbreviations: CNBr, cyanogen bromide; DMF, dimethylformamide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; F_1, catalytic sector (soluble) of the beef heart mitochondrial ATPase; FDNP, 1,5-difluoro-2,4-dinitrobenzene; FSBI, 5′-[*p*-(fluorosulfonyl)benzoyl]inosine; MES, 2-(*N*-morpholino)ethanesulfonic acid; NANDP, 2-[(4-azido-2-nitrophenyl)amino]ethyl diphosphate; NDP, nucleotide diphosphate; SDS, sodium dodecyl sulfate; TDAB, tetradecyltrimethylammonium bromide; TEA-HCO_3, triethylammonium bicarbonate; TFA, trifluoroacetic acid.

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AMP was from Amersham. All the chemical reagents were of the highest purity available. Those utilized to synthesize 2-azido[^{3}H]ADP and 8-azido[^{3}H]ADP were redistilled.

Synthesis of 2-Azido[^{3}H]ADP. A 30-mg portion of 2-chloroadenosine was added to 10 mCi of 2-chloro[8-^{3}H]adenosine and diazotized with 900 μL of hydrazine (Schaeffer & Thomas, 1958). 2-Azido[^{3}H]adenosine was first converted to 2-azido-[^{3}H]AMP according to the method of Sowa and Ouchi (1975). 2-Azido[^{3}H]AMP was purified with a Dowex 50W-X$_4$H$^+$ (Boulay et al., 1985) and phosphorylated into 2-azido[^{3}H]-ADP by the method of Hoard and Ott (1965) using carbonyldiimidazole as the coupling reagent. 2-Azido[^{3}H]-ADP was purified by chromatography on a DE 52 column (Whatman) that was eluted with a 2-L linear gradient of 0–0.5 M TEA-HCO_3 (pH 7.4) (Czarnecki et al., 1979). An 8-μmol sample of 2-azido[^{3}H]ADP was obtained. The specific activity of the final product was 210 dpm/pmol.

Synthesis of 8-Azido[^{3}H]ADP. 8-Br[2-^{3}H]AMP was synthesized as described by Czarnecki et al. (1979). A 12-mg portion of AMP was added to 5 mCi of [2-^{3}H]AMP and converted to 8-Br[2-^{3}H]AMP with bromine water. The gumlike triethylammonium salt of 8-Br[2-^{3}H]AMP (15 μmol) was dried twice with anhydrous DMF in a rotary evaporator. This was followed by addition of 150 μL of a solution of 1 M tetraethylammonium azide in anhydrous DMF, resulting in the synthesis of 8-azido[^{3}H]AMP (Boos et al., 1978). After one night at 70 °C, the reaction mixture was applied to a DE 52 column. Elution was carried out with a linear gradient of 0–0.4 M TEA-HCO_3 (pH 7.4), 1-L total volume. The presence of 8-azido[^{3}H]AMP in the eluates was characterized spectrophotometrically by absorbance at 281 nm (Boos et al., 1978). For phosphorylation of 8-azido[^{3}H]AMP to 8-azido-[^{3}H]ADP, the same protocol as that described for 2-azido-[^{3}H]ADP was used. A 12-μmol sample of purified 8-azido-[^{3}H]ADP was obtained with a specific activity of 520 dpm/pmol.

Preparation of Mitochondrial F_1. Beef heart mitochondria were prepared as described by Smith (1967). Beef heart mitochondrial F_1 was prepared according to the method of Knowles et al. (1972) modified by Klein et al. (1982), and stored at 4 °C, as an ammonium sulfate precipitate. Prior to use, the enzyme suspension was pelleted. The pellet was rinsed with 60% (w/v) ammonium sulfate in a medium composed of 50 mM Tris–MES and 5% (w/v) glycerol (TMG medium), and then solubilized in the same buffer without ammonium sulfate. It was immediately subjected to two sequential filtration–centrifugations through 2-mL Sephadex G50 columns [adapted from Penefsky (1977)]. The first column was equilibrated with the solubilization medium, and the second one was equilibrated with the solubilization medium supplemented with 2 mM $MgCl_2$ (TMMG medium). This treatment yielded a mitochondrial F_1 preparation essentially free of the three loosely bound nucleotides present on the native enzyme, but still containing three tightly bound nucleotides.

Assay of ATPase Activity. The ATPase activity was measured spectrophotometrically at pH 7.5 and at 30 °C, using a standard regenerating system containing 200 μM NADH, 2 mM phosphoenolpyruvate, 20 μg/mL pyruvate kinase, 10 μg/mL lactate deshydrogenase, 2.5 mM ATP, 2 mM $MgCl_2$, 50 mM KCl, and 100 mM Tris–H_2SO_4. It was checked that $AlCl_3$ and NaF had no effect on the regenerating system under our experimental conditions.

Trapping of ADP and Azido-ADP by F_1. Desalted F_1 was preincubated at the concentration of 5 μM in dim light with [^{3}H]ADP (100 μM), 2-azido[^{3}H]ADP (100 μM), or 8-azido-[^{3}H]ADP (100 μM), for 30 min at 37 °C. Inhibition was initiated by addition of 5 mM NaF and 200 μM $AlCl_3$. At various times, samples were withdrawn, subjected to centrifugation–filtration in TMG medium to remove free ligands, then submitted to a cold chase in the presence of 5 mM ATP for 10 min, and finally precipitated by ammonium sulfate. After 30 min at 4 °C, the pellets recovered by centrifugation were solubilized in TMG buffer and the solution was filtered through a second G50 column equilibrated in the same buffer. Aliquots of the eluted material were used for measurement of ATPase activity and determination of bound radioactive nucleotides. The labeled eluates were pooled and further used for photolabeling. Radioactivity was measured by scintillation counting with Ready Value Cocktail (Beckman). The protein concentration was estimated using the Coomassie Blue method (Bradford, 1976).

Photoaffinity Labeling of Inhibited F_1

Purification and Fragmentation of the Photolabeled β Subunit. Photoactivation was carried out on 10 mg of inhibited F_1 containing the entrapped azido-nucleotides due to the presence of NaF and $AlCl_3$. The source of light was a Xenon XB 100 lamp (1000 W) equipped with a parabolic reflector. Photoirradiation lasted for 25 s. The photolabeled F_1 was recovered as an ammonium sulfate precipitate, solubilized, and dissociated into subunits using a medium consisting of 50 mM sodium succinate, 1 M sodium chloride, 0.25 M sodium nitrate, 0.1 mM dithiothreitol, and 4 mM EDTA (Issartel et al., 1983). The photolabeled β subunit was purified by chromatography on DE-52 cellulose (Whatman), succinylated, and then fragmented by cyanogen bromide and trypsin. The method used was essentially similar to that adopted for exploring the region of the β subunit photolabeled by 2-azido-[^{32}P]ADP and azido[^{32}P]Pi (Garin et al., 1986 & 1989). The resulting peptides were fractionated by gel filtration (Sephadex G75 column, Pharmacia, and Bio-Gel P6 column, BioRad) or by reversed-phase HPLC (C_4 column, Vydac). The nomenclature of peptides resulting from chemical or enzymatic cleavage of the β subunit was that adopted by Runswick and Walker (1983).

Identification of the Photolabeled Amino Acid Residues. Prior to sequencing, peptide R_{20} of the β subunit was covalently immobilized on Sequelon arylamine disks (Millipore). Briefly, a disk of Sequelon arylamine was placed on a piece of aluminum foil. A 5-μL aliquot of the purified R_{20} solution (0.1 nmol/μL) was applied on the disk, and allowed to evaporate at 37 °C. This was followed by addition of another 5-μL aliquot which was also evaporated. The total applied radioactivity was about 40 000 cpm, corresponding to 2 nmol of peptide R_{20}. At last, 5 μL of TFA (100%) was spotted on the disk and evaporated to ensure an acidic pH for the EDC coupling. This disk was wetted with 5 μL of 0.1 M MES (pH 5.0) containing 15% acetonitrile and 10 mg/mL EDC, and placed in a small closed Eppendorf tube containing 10 μL of H_2O at 37 °C for 15 h. Noncovalently bound peptide was removed by two 1-mL TFA (100%) washes. The coupling yield was 85%. The disk was then placed in the protein sequencer (Applied Biosystem 477A coupled to an HPLC 120A chromatography system). Radioactivity elution was optimized by introducing extensive washing of the Sequelon membrane with TFA in the sequencer program to avoid excessive carry-over from one amino acid residue to the next.

RESULTS

In the Presence of NaF and $AlCl_3$, 2-Azido[^{3}H]ADP and 8-Azido[^{3}H]ADP Are Entrapped into the F_1 Nucleotide Binding Sites. As previously shown, mitochondrial F_1-ATPase

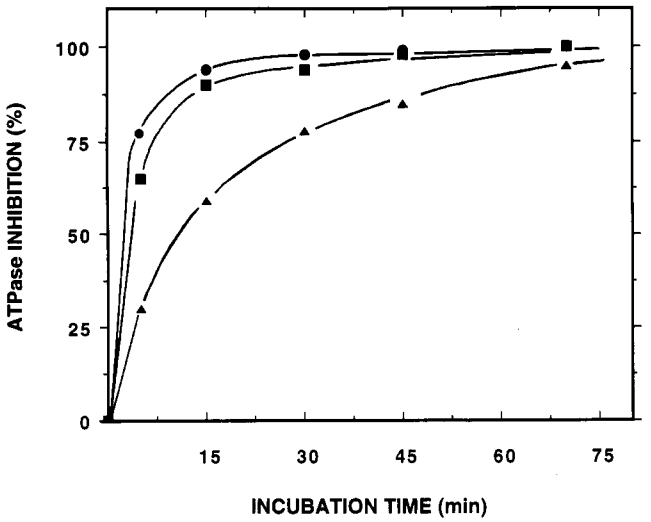

FIGURE 1: Time dependence of the inhibition of F_1-ATPase activity in the presence of ADP (●), 2-azido-ADP (■), or 8-azido-ADP (▲). Desalted F_1 (5 μM) was preincubated in TMMG medium (see Materials and Methods) for 30 min at 30 °C in dim light and in the presence of 100 μM [^{14}C]ADP, 100 μM 2-azido[^{3}H]ADP, or 100 μM 8-azido[^{3}H]ADP. Sodium fluoride and aluminum chloride were further added at final concentrations of 5 mM and 200 μM, respectively. At various times, samples were assayed for their ATPase activity. Control F_1 was treated in the same way except that aluminum chloride was omitted.

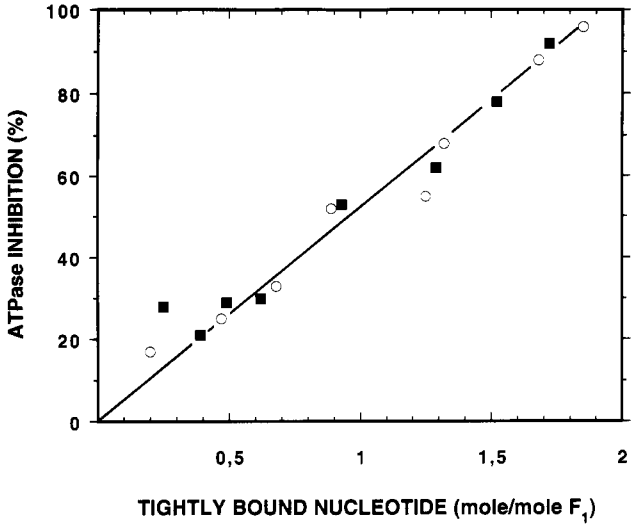

FIGURE 2: Correlation between the tightly bound nucleotides and the inhibition by fluoroaluminate. Desalted F_1 (1 μM) was incubated in dim light for 30 min at 37 °C in TMMG medium containing 50 μM 2-azido[^{3}H]ADP (○) or 8-azido[^{3}H]ADP (■) and 5 mM sodium fluoride. Inhibition was initiated by the addition of 100 μM aluminum chloride. At various times, samples were withdrawn, freed of loosely bound ligands as described under Materials and Methods, and assayed for bound nucleotide and ATPase activity.

activity is fully and quasi irreversibly inhibited by fluoroaluminum or fluoroberyllium complexes provided ADP is present in the medium (Lunardi et al., 1988). Very tight binding of ADP to F_1 appeared to be responsible for the enzyme inhibition which developed in a time-dependent manner, suggesting a slow transition of the enzyme from an active conformation to an inactive one (Lunardi et al., 1988; Dupuis et al., 1989; Issartel et al., 1991). Figure 1 shows that if, in dim light, ADP is replaced by 2-azido[^{3}H]ADP or 8-azido-[^{3}H]ADP, inhibition also develops. When $MgCl_2$, $AlCl_3$, NaF, or the azido-nucleotide was omitted, inhibition did not occur (not shown). When the added nucleotide was 8-azido[^{3}H]-ADP, half-inhibition was attained in about 10 min. In the cases of [^{3}H]ADP and 2-azido[^{3}H]ADP, the half-time was only 1 and 2 min, respectively (Figure 1). This higher efficiency of 2-azido-ADP or ADP compared to 8-azido-ADP is likely attributable to differences in the nucleotide conformations. In fact, 2-azido-ADP in solution, like ADP, predominantly adopts an *anti* conformation (Czarnecki, 1984) in contrast to 8-azido-ADP which is mainly in the *syn* conformation (Sarma et al., 1974). As nucleotides bind to the F_1 catalytic sites in the *anti* conformation (Garin et al., 1988), the equilibrium between the *syn* and *anti* conformations of 8-azido-ADP might be, under our experimental conditions, a limiting factor in the nucleotide entrapment and the subsequent inhibition.

In dim light, the stoichiometry of tightly bound 2-azido-[^{3}H]ADP or 8-azido[^{3}H]ADP was determined together with the extent of inhibition of the F_1-ATPase activity (Figure 2). Inhibition of ATPase activity was linearly related to the tight binding of azido[^{3}H]ADP, full inhibition being attained for about 1.9 mol of bound azido[^{3}H]ADP/mol of F_1. This result points to the similar behavior of azido[^{3}H]ADP and ADP with regards to the fluorometal inhibition.

Upon Photoirradiation of F_1 in the Presence of 2-Azido[^{3}H]-ADP or 8-Azido[^{3}H]ADP and $AlCl_3$ and NaF, Only the β Subunit Is Labeled. Prior to photoirradiation, F_1 was incubated with NaF and $AlCl_3$ in the presence of either 2-azido-[^{3}H]ADP or 8-azido[^{3}H]ADP in dim light, and inhibition was allowed to develop till completion. The nontightly bound nucleotides were removed, and photoirradiation was carried out (see Materials and Methods). The photolabeled F_1 was dissociated into its constitutive subunits by treatment with a nitrate medium (see Materials and Methods). The released subunits were resolved by chromatography on DE-52 cellulose. With both 2-azido[^{3}H]ADP and 8-azido[^{3}H]ADP, about 90% of the radioactivity was recovered in a protein peak corresponding to the β subunit. As revealed by TDAB–polyacrylamide gel electrophoresis, and in agreement with preceeding studies (Issartel et al., 1983; Garin et al., 1986, 1989), the 10% radioactivity found in the αγδε peak was due to contaminant β subunit. It is therefore concluded that the β subunit of F_1 recognizes specifically the two photoprobes. The yield of photolabeling was calculated from the amounts of tightly bound azido probe in the dark and covalently bound azido probe following photoirradiation. The amount of covalently bound 2-azido[^{3}H]ADP was 0.5 mol/mol of purified β subunit, starting from a F_1 preparation containing 1.8 mol of entrapped 2-azido[^{3}H]ADP/mol of F_1, *i.e.*, 0.6 mol of entrapped 2-azido[^{3}H]ADP/mol of β subunit. The deduced yield of photolabeling was 80%, which is an unusually high value. In the case of photolabeling by 8-azido[^{3}H]ADP in the presence of fluoroaluminate, the yield was close to 50%. By comparison, in the absence of fluorometal, the photolabeling yields were 25% for 2-azido[^{3}H]ADP and 20% for 8-azido-[^{3}H]ADP.

Upon Photoirradiation, Entrapped 2-Azido[^{3}H]ADP and 8-Azido[^{3}H]ADP Label the Same β-Tyr-345 Amino Acid Residue. The purified photolabeled β subunit (about 40 nmol) was succinylated and submitted to CNBr cleavage at the methionyl residues as described in the Materials and Methods. The resulting peptide fragments were fractionated by gel filtration on a Sephadex G75 column (Figure 3). The profile of radioactivity corresponding to the elution of the peptide fragments from the photolabeled β subunit was virtually the same with 2-azido[^{3}H]ADP or 8-azido[^{3}H]ADP used as photoprobes. For the sake of conciseness, only the results obtained with 8-azido[^{3}H]ADP will be presented. As shown in Figure 3, one major radioactive peak, referred to as G2 and containing about 85% of the radioactivity, was eluted. It was

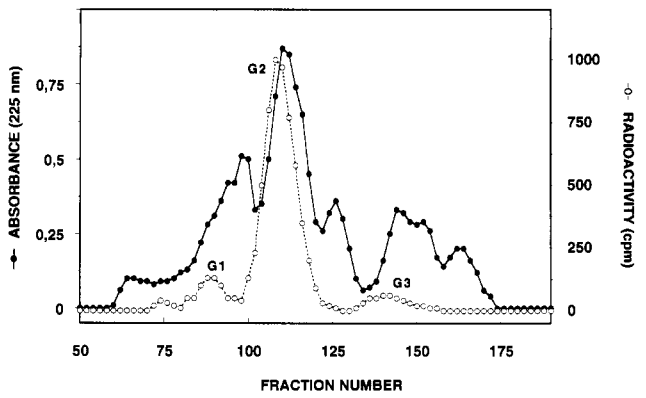

FIGURE 3: Fractionation of CNBr cleavage products of photolabeled β subunit. The resulting peptides were chromatographed on a Sephadex G75 SF column (120 × 2 cm) in 50 mM ammonium bicarbonate. During elution, 3-mL fractions were collected and analyzed for absorbancy at 225 nm and radioactivity (20-μL aliquots were counted). Fractions 100–120 were pooled. They corresponded to 85% of the eluted radioactivity. The recovery of the radioactive material was about 95%.

accompanied by two minor radioactive peaks, G1 and G3. SDS–PAGE revealed that peak G1 contained high molecular weight peptides, which arose from incomplete cleavage of the β subunit by CNBr (not shown). The material of peak G2 was concentrated and subjected to reversed-phase HPLC. The radioactivity was eluted as a single peak (Figure 4A) which was identified by N-terminal sequence analysis as peptide CB9, spanning Gln-293–Met-358. Radioactivity eluted with peak G3 was associated with a peptide referred to as CB'9 (Garin et al., 1986). CB'9 was derived from CB9 by acidolytic cleavage at the Asp-319–Pro-320 bond during the course of the CNBr attack in 80% formic acid.

For a better resolution of the photolabeled region of the β subunit, peptide CB9 was further cleaved by trypsin, and the tryptic digest was fractionated by gel filtration on a Bio-Gel P-6 column (Figure 4B). In the elution profile, the radioactivity was associated with fractions 42–50. These fractions were concentrated, and the corresponding peptide (R_{20}) was covalently immobilized on a Sequelon arylamine disk for sequence analysis (see Materials and Methods). Radioactivity was released only at step 8, corresponding to β-Tyr-345 (Figure 5). Similar results were obtained with 2-azido[^{3}H]ADP (not shown).

DISCUSSION

Inhibition of F_1-ATPase activity by ADP in the presence of fluoroaluminate or fluoroberyllate complexes was first reported by Lunardi et al. (1988). Because GDP and IDP were as effective as ADP in supporting ATPase inhibition, and are known to bind only to the F_1 catalytic sites, it was assumed that inhibition was due to the formation of tight NDP–fluoroaluminate complexes within the catalytic sites. As the amino acid residue β-Tyr-345 is thought to be a constitutive part of the F_1 catalytic sites [for reviews, see Allison et al. (1992) and Duncan and Cross (1992)], the results presented here further support the idea that F_1 inhibition by ADP–fluoroaluminate complexes results from the very tight binding of nucleotide diphosphate to the catalytic sites of F_1.

The Azido[^{3}H]ADP–Fluorometal Complex Binds More Efficiently than Azido[^{3}H]ADP to the Catalytic Site of F_1. In photolabeling experiments, the demonstration of the valid use of a given probe implies a number of controls to be carried out in dim light to assess the affinity and specificity of the photoprobe by comparison with those of the natural ligand (Czarnecki et al., 1979; Chowdhry & Westheimer, 1979; Vignais & Lundardi, 1985). In fact, it may happen that the photoprobe binds to amino acid residues not exactlty located in the catalytic site. This is due to the summation of a number of parameters, including a certain degree of freedom of the probe in the catalytic crevice, the exchangeability of the ligand, the long half-life time of the photogenerated nitrene groups, and the possible chemical selectivity of the nitrene for some reactive amino acid residues (Chowdhry & Westheimer, 1979; Staros, 1980; Brunner, 1981). For these reasons, the virtually irreversible binding of azido[^{3}H]ADP in the presence of fluoroaluminate to the F_1 catalytic sites was particularly advantageous for mapping studies. A similar situation was recently encountered in the case of myosin, with the entrapment of 2-azido-ATP at the active site by complexation of two reactive thiols with cobalt(III) phenanthroline (Grammer et al., 1993). In the case of F_1, the entrapment of the photoprobes

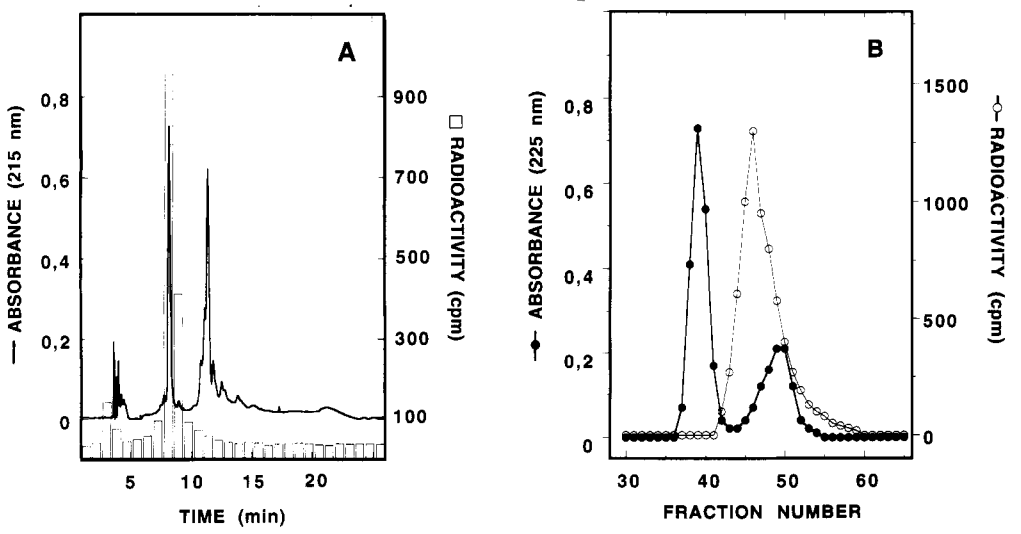

FIGURE 4: (A) Purification of the major radioactive peptide obtained by CNBr cleavage by HPLC. The column (C4, 10 μm, 4 mm × 200 mm) was equilibrated in 75% buffer A (0.1% TFA) and 25% buffer B (0.1% TFA, 80% CH_3CN). The material corresponding to the pooled G2 fractions (Figure 3) was injected, and the column was eluted at a flow of 1 mL min^{-1}. Fractions of 10 μL were collected at 1-min intevals, and analyzed for their radioactivity content. The radioactivity recovery was 85%. (B) Fractionation of the trypsin digest of photolabeled CB9 by gel chromatography. Purified CB9 peptide in 100 mM ammonium bicarbonate was digested by trypsin (1/100, w/w) for 18 h at 37 °C. The digest was chromatographed on a Bio-Gel P6 column (1.5 × 100 cm) equilibrated in 100 mM ammonium bicarbonate. Fractions of 1 mL were collected, from which 20 μL was withdrawn for determination of radioactivity.

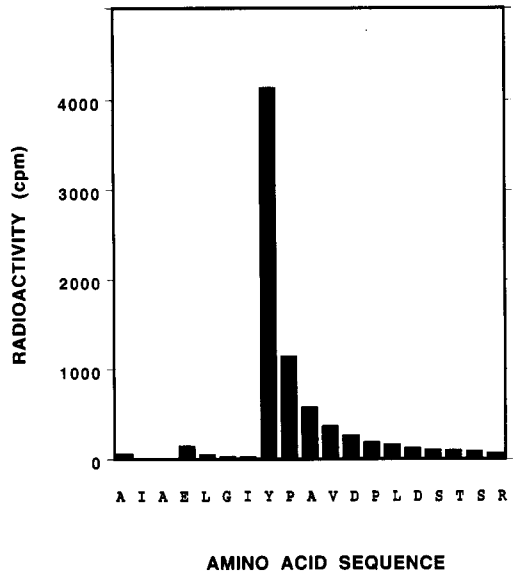

FIGURE 5: Radioactivity elution from the Edman degradation of the 8-azido[^{3}H]ADP-labeled R_{20}. A fraction of the peptide, containing 40 000 cpm, was spotted onto the arylamine Sequelon filter and covalently linked via EDC coupling (see Materials and Methods). The derivatized amino acid obtained at each cycle of Edman degradation was counted for radioactivity. The amino acid sequence of the peptide is indicated along the *x* axis. The data in the figure are not corrected for sequencing repetitive yield.

in the presence of fluorometal resulted in a significant increase in the photolabeling yields by comparison with the photolabeling carried out in the absence of fluorometals. The increased yield is most likely due to the immobilization of the azido-probe in the catalytic crevice, thus resulting in the shortening of distances between the probe and the interacting amino acid residues. Immobilization of the probe also explains the restriction of the labeling to one given amino acid residue, namely, β-Tyr-345.

New Insights into the Structure of the F_1 Catalytic Sites. The effect of fluorometal on photolabeling of F_1 subunits was of particular interest in the case of 8-azido[^{3}H]ADP. In the absence of fluorometal, about 25% of the photolabeling was associated with the F_1 α subunit (Hollemans et al. (1983) and personal results). By contrast, when photolabeling was performed with 8-azido[^{3}H]ADP in the presence of fluoroaluminate as described here, only the β subunit was radiolabeled, like in the case of F_1 photolabeled with 2-azido[^{3}H]-ADP (Garin et al., 1986). Photolabeling of the α subunit by 8-azido-ADP/ATP in the absence of fluorometal might be explained by interaction of the photoprobe with noncatalytic sites or by the localization of the catalytic sites at the αβ interface (Sloothaak et al., 1985; Van Dongen & Berden, 1986). It is also possible that the α subunit becomes labeled during the "in" and "out" movements of the photoactivated probe from the β catalytic site.

The fact that β-Tyr-345 is the only amino acid residue labeled by both 2-azido[^{3}H]ADP and 8-azido[^{3}H]ADP in the presence of fluoroaluminate indicates that an important conformational change occurs when the catalytic sites of F_1 bind NDP–fluorometal complexes. As a matter of fact, it should be recalled that β-Ile-304 and β-Tyr-311 were identified as the amino acid residues of F_1 which are photolabeled by 8-azido[^{14}C]ATP in the absence of fluorometal (Hollemans et al., 1983). In a control experiment, conducted with 8-azido-[^{3}H]ADP in the absence of fluoroaluminate, we confirmed this result except that photolabeling was restricted to β-Tyr-311. The "labeling shift" from β-Tyr-311 to β-Tyr-345 promoted by fluorometal favors the idea of a conformational change which affects the F_1 spatial structure. No labeling shift was observed in the case of 2-azido[^{3}H]ADP labeling which always occurs on β-Tyr-345.

The labeling of the same β-Tyr-345 amino acid residue by 2-azido[^{3}H]ADP and 8-azido[^{3}H]ADP entrapped into the F_1 catalytic sites remains to be explained. The distance between C2 and C8 in the purine ring is approximately 6 Å, making it plausible that the azido groups at the C2 and C8 of the adenine ring react with different amino acid residues of the catalytic crevice. This was indeed the case when photolabeling was carried out in the absence of fluorometal (Hollemans et al., 1983; Garin et al., 1986). A possible explanation for the photolabeling of the same β-Tyr-345 amino acid residue with either entrapped 2-azido-ADP or 8-azido-ADP is that the two probes in the catalytic crevice have some rotational freedom. The photogenerated nitrene would then bind to the amino acid residue of the crevice which would be the more reactive. However, recent photolabeling data concerning the myosin ATP binding site do not favor this hypothesis. In fact, in rabbit skeletal myosin, Trp-130 is labeled by 2-azido-ATP and NANDP (Grammer et al., 1993; Okamoto & Yount, 1985), and in scallop myosin, in which Trp-130 is replaced by an Arg amino acid residue, Arg-130 is selectively photolabeled (Kerwin & Yount, 1992). Thus, photolabeling of amino acid residues in myosin seems to be independent of their chemical reactivity. Another hypothesis would be that the conformational change which accompanies the ADP–fluorometal inhibition of F_1 first would move the β-Tyr-311 region away from the adenine ring of the tightly bound nucleotide, and second would bring β-Tyr-345 close to the adenine ring of the nucleotide in such a way that this residue would be the only one to be labeled by either 2-azido[^{3}H]ADP or 8-azido[^{3}H]-ADP. This hypothesis would be in agreement with previous results obtained by site-directed mutagenesis on *Escherichia coli* F_1-ATPase (Wise, 1990), and with the recent finding that the phenolic hydroxyl of the β-Tyr-331 of *E. coli* F_1, homologous to beef heart F_1 β-Tyr-345, directly interacts with the fluorophore of *lin*-benzo-ADP (Weber et al., 1992).

One of the regions involved in the F_1 catalytic site of the β subunit is the so-called "P-loop motif" ($_{156}$GXXXXGKT$_{163}$). The P-loop motif is present in a variety of purine nucleotide binding proteins (Walker et al., 1982; Saraste et al., 1990). In the case of adenylate kinase, a conformational change of the P-loop was observed in two crystal forms of the enzyme, and was postulated to correspond to the transition from a blocked form to an opened form of the MgATP binding site (Fry et al., 1986). The P-loop region might be close to the β-Tyr-311 region (Garin et al., 1989). By analogy with other nucleotide binding proteins, it is assumed that the P-loop region of the β subunit of F_1 interacts with the polyphosphate chain of the bound nucleotide (Duncan & Cross, 1992; Futai et al., 1992). As F_1 inhibited by ADP–fluoroaluminate is supposed to be blocked in a conformation analogous to that of the transition state of the enzyme, with ADP–Pi trapped in a closed catalytic site (Dupuis et al., 1989; Issartel et al., 1991), the tightly bound fluoroaluminate is expected to interact with the P-loop region of the β subunit. Because of this interaction, and by analogy with adenylate kinase, it is reasonable to suggest that the P-loop region might constitute a turning point for the ADP–fluoroaluminate-induced conformational change of F_1, and therefore for conformational changes of F_1 which occur during catalysis. The recent analysis of cross-linking products obtained between the β and γ subunits of *E. coli* F_1-ATPase would be in favor of this hypothesis. It was shown, with a new bifunctional reagent, that the P-loop region can be cross-linked to the γ subunit of F_1 when MgADP is bound in the

catalytic site. This cross-link cannot form when ADP is replaced by ATP (Aggeler et al., 1993).

In conclusion, inhibition of the mitochondrial F_1-ATPase activity by the ADP–fluorometal complex is accompanied by a major conformational change of the enzyme catalytic site that is reflected by a different reactivity of 2-azido-ADP and 8-azido-ADP toward specific amino acid residues. As ATP and GTP binding proteins with trapped NDP–fluorometal are believed to adopt a conformation close to that exhibited in the transition state during the normal course of the catalysis (Bigay et al., 1987; Carlier et al., 1988; Lunardi et al., 1988; Dupuis et al., 1989; Combeau & Carlier, 1988, 1989; Maruta et al., 1991; Garin & Vignais, 1993), the detailed study of this new conformation should be of interest with respect to the catalytic mechanism of the enzyme.

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