Registry No. Na^+, 7440-23-5; H^+, 12408-02-5.

References

Bakker, E. P., & Mangerich, W. E. (1983) *Biochim. Biophys. Acta 730*, 379–386.

Bassilana, M., Damiano, E., & Leblanc, G. (1984) *Biochemistry 23*, 1015–1022.

Beck, J. C., & Rosen, B. P. (1979) *Arch. Biochem. Biophys. 194*, 208–214.

Booth, I. R. (1981) *Trends Biochem. Sci. (Pers. Ed.) 70*, 257–261.

Brey, R. N., Beck, J. C., & Rosen, B. P. (1978) *Biochem. Biophys. Res. Commun. 83*, 1588–1594.

Brey, R. N., Rosen, B. P., & Sorensen, E. N. (1980) *J. Biol. Chem. 255*, 39–44.

Cohen, G. N., & Rickenberg, H. V. (1956) *Ann. Inst. Pasteur, Paris 91*, 693–720.

Damiano, E., Bassilana, M., Rigaud, J. L., & Leblanc, G. (1984) *FEBS Lett. 166*, 120–124.

Harold, F. M., & Altendorf, K. (1974) *Curr. Top. Membr. Transp. 5*, 1–50.

Kaback, H. R. (1971) *Methods Enzymol. 22*, 99–120.

Kaback, H. R., & Barnes, E. N. (1971) *J. Biol. Chem. 246*, 5523–5531.

Kaczorowski, G. J., Robertson, D. E., & Kaback, H. R. (1979) *Biochemistry 18*, 3697–3704.

Komor, E., Schwab, W. G., & Tanner W. (1979) *Biochim. Biophys. Acta 555*, 524–530.

Lancaster, J. R., & Hinkle P. (1977) *J. Biol. Chem. 252*, 7657–7661.

Lanyi, J. K. (1979) *Biochim. Biophys. Acta 559*, 377–398.

Lanyi, J. K., & Silverman, M. P. (1979) *J. Biol. Chem. 254*, 4750–4755.

Leblanc, G., Rimon, G., & Kaback, H. R. (1980) *Biochemistry 19*, 2522–2528.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mitchell, P. (1968) in *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, England.

Mitchell, P. (1970) in *Membranes and Ion Transport* (Bittar, E. E., Ed.) Vol. 1, pp 192–256, Wiley-Interscience, New York.

Mitchell, P. (1973) *J. Bioenerg. 4*, 63–91.

Padan, E., Zilberstein, D., & Schuldiner, S. (1981) *Biochim. Biophys. Acta 650*, 151–166.

Reenstra, W. W., Patel, L., Rottenberg, H., & Kaback, H. R. (1980) *Biochemistry 19*, 1–9.

Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17*, 706–711.

West, I. C., & Mitchell, P. (1974) *Biochem. J. 144*, 87–90.

Zilberstein, D., Schuldiner, S., & Padan, E. (1979) *Biochemistry 18*, 669–673.

Photolabeling on β-Subunit of the Nucleotide Site Related to Hysteretic Inhibition of Mitochondrial F_1-ATPase†

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ABSTRACT: While F_1-ATPase can hydrolyze about any nucleoside triphosphate, it can undergo a hysteretic inhibition only in the presence of nucleotides or analogues bearing an adenine ring [Baubichon, H., Godinot, C., Di Pietro, A., & Gautheron, D. C. (1981) *Biochem. Biophys. Res. Commun. 100*, 1032–1038]. This difference in specificity has been used to identify the location of the regulatory site in F_1-ATPase. 3′-*O*-[3-[*N*-(4-Azido-2-nitrophenyl)amino]propionyl]adenosine 5′-diphosphate (NAP_3-ADP) behaves as ADP to induce the hysteretic inhibition of F_1-ATPase. The radioactive analogue also binds to F_1-ATPase with the same stoichiometry and the same concentration dependence as ADP. It is therefore an excellent photoaffinity label to localize the regulatory site. Catalytic sites being occupied by guanosine 5′-(β,γ-imidotriphosphate), the photoirradiation-induced covalent binding of NAP_3-ADP to the β-subunit of F_1-ATPase can be directly related to the hysteretic inhibition. On the contrary, there is no correlation between the inhibition of ATPase activity and the limited binding of NAP_3-ADP to the α-subunit. It is therefore concluded that the regulatory site must be located on the β-subunit of the mitochondrial F_1-ATPase.

Previous studies from our laboratory (Di Pietro et al., 1980) have shown that preincubation of pig heart mitochondrial F_1-ATPase with ADP[1] leads to binding of ADP, which induces a progressive hysteretic inhibition of MgATP hydrolysis. Since this binding was Mg dependent and since it was reversed by ammonium sulfate precipitation, this ADP was not "tightly bound" as defined by Harris et al. (1978). This binding occurs at regulatory site(s) specific to adenine nucleotides (Baubichon et al., 1981; Di Pietro et al., 1981). On the contrary, all nucleoside triphosphates can be hydrolyzed at the catalytic site(s) (Schuster et al., 1975; Pedersen, 1975; Harris et al., 1978). The aim of this study is to use this difference in specificity of catalytic and regulatory sites to localize the

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[1] Abbreviations: ATPase, adenosine-5′-triphosphatase; F_1-ATPase, pig heart mitochondrial F_1-ATPase purified according to the procedure of Penin et al. (1979), omitting the last step (gel filtration in the presence of 50% glycerol); ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GMP-P(NH)P, guanosine 5′-(β,γ-imidotriphosphate); NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; NAP_3, [*N*-(4-azido-2-nitrophenyl)amino]propionyl; NAP_3-ADP or arylazido-β-alanyl-ADP, 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl]adenosine 5′-diphosphate; TDAB, tetradecyltrimethylammonium bromide; FSBA, [*p*-(fluorosulfonyl)benzoyl]adenosine.

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regulatory site(s) by using a covalent ADP analogue. Any modification on the adenine ring prevented the setting up of the hysteretic inhibition. Among analogues not modified on the adenine moiety, Russell et al. (1976) have shown that arylazido-β-alanyl-ATP is a good substrate for F_1-ATPase in the dark and can be covalently bound to F_1-ATPase by photoirradiation. Moreover, Lunardi et al. (1981) have covalently bound an arylazido-β-aminobutyryl-ADP to the α- and β-subunits of the F_1-ATPase. Therefore, the use of an arylazido-ADP seemed to be well suited for the localization of ADP sites.

This paper shows that arylazido-β-alanyl-ADP (NAP_3-ADP) exactly mimicks the effects of ADP in inducing the hysteretic inhibition and the concomitant nucleotide binding. When the hydrolytic sites were occupied by GMP-P(NH)P, [^{3}H]NAP_3-ADP was bound preferentially to the β-subunit rather than to the α-subunit. Moreover, the incubation of the enzyme with ADP under conditions inducing the hysteretic inhibition, prior to the addition of [^{3}H]NAP_3-ADP, essentially decreased the radioactivity incorporated in the β-subunit. These experiments lead to the conclusion that the binding of NAP_3-ADP on the β-subunit is responsible for the hysteretic inhibition.

Experimental Procedures

Materials. Nucleotides were purchased from Boehringer Mannheim. [2,8-^{3}H]ADP (29.7 Ci/mmol) came from the Radiochemical Centre, Amersham, England. Their purity was checked by thin-layer ascending chromatography on poly(ethylenimine)–cellulose with 0.7 M LiCl as the developing solvent. Arylazido-β-alanyl-ADP was prepared according to Guillory & Jeng (1977). Arylazido-β-alanyl-[^{3}H]ADP was prepared by condensing [2,8-^{3}H]ADP with 3-[*N*-(4-azido-2-nitrophenyl)amino]propionic acid. The reaction products were resolved by thin-layer chromatography on cellulose (Merck) with 1-butanol–water–acetic acid (5:3:2) as eluent. The spectral characteristics were identical with those described by Guillory & Jeng (1977).

Enzyme Preparations. Pig heart mitochondria were obtained at 0–4 °C as previously described (Gautheron et al., 1964). The mitochondrial F_1-ATPase was purified by the method of Penin et al. (1979), omitting the last step (gel filtration in the presence of 50% glycerol) that removed the tightly bound nucleotides. The F_1-ATPase obtained contained 1.8 ± 0.2 mol of ADP and 0.3 ± 0.03 mol of ATP/mol. It was stored at 0–4 °C as an ammonium sulfate suspension in 50 mM Tris–H_2SO_4, pH 7.8, containing 2 mM EDTA and 4 mM ATP. Just before use, an aliquot was centrifuged at 9000*g* for 5 min. The pellet was dissolved in 50 mM Tris–H_2SO_4, pH 8.0, containing 10% glycerol at room temperature. The enzyme was precipitated twice with 60% ammonium sulfate to remove nucleotides that were not tightly bound. The enzyme was equilibrated with 50 mM Tris–H_2SO_4, pH 8.0, 10% glycerol, and 1 mM EDTA by filtration–centrifugation, following the procedure described by Penefsky (1977).

The protein content of the enzyme solution was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The molecular weight of F_1-ATPase was taken as 380 000 (Di Pietro et al., 1975).

Assay of ATPase Activity. The measurements were performed on 0.5–4-μL aliquots at 30 °C in 0.62 mL of 50 mM Tris–H_2SO_4 buffer, pH 8.0, 3.3 mM ATP, and 3.3 mM $MgSO_4$ with an ATP-regenerating system (phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, and NADH) and by following with a spectrophotometer the rate of NADH disappearance at 340 nm [Pullman et al. (1960) as modified by Baubichon et al. (1982)]. The specific ATPase activity measured in the presence or the absence of 10 mM (sodium) bicarbonate as an activating anion was respectively 65–80 or 35–40 μmol of ATP hydrolyzed min^{-1} mg^{-1}.

Measurements of NAP_3-ADP-Induced or ADP-Induced Hysteretic Inhibitions and Concomitant ^{3}H-Labeled Nucleotide Binding. After incubation of F_1-ATPase (1 mg of protein/mL) in 50 mM Tris–H_2SO_4–1.5 mM $MgSO_4$–10% glycerol, pH 8.0, for 30 min, an aliquot (1–2 μL) was transferred into the spectrophotometer cuvette to measure the ATPase activity as above. ^{3}H-Labeled nucleotide (200 μM) was added and incubated for 30 min in the dark. The ADP-induced hysteretic inhibition and the concomitant ADP binding were measured as described previously (Di Pietro et al., 1980, 1981).

Photolabeling of F_1-ATPase with [^{3}H]NAP_3-ADP. F_1-ATPase (0.5–1 mg of protein/mL) was preincubated in the dark for 30 min at 30 °C in 50 mM Tris–H_2SO_4, pH 8.0, 10% glycerol, and 1.5 mM $MgSO_4$ with or without 0.2 mM ADP. [^{3}H]NAP_3-ADP was first added, and GMP-P(NH)P (0.5 mM) was added 10 min later. The ATPase activity was measured on 1–2-μL aliquots as above. A sample of 0.1 mL was introduced in a 1.5-mL Eppendorf microcentrifuge tube that was held in a water-jacketed cell maintained at 30 °C with a circulating water bath. The cell was illuminated with visible light from a 250-W Osram lamp (cooled with an air blower) situated 10 cm from the tubes, which were covered with a filter cutting off the radiations below 300 nm. After 30–90 min of irradiation, free nucleotides were removed by filtration–centrifugation through a 1-mL tuberculin syringe containing Sephadex G-50 (fine) swollen in 10% glycerol–50 mM Tris–H_2SO_4, pH 8.0, following the procedure of Penefsky (1977). Since this filtration–centrifugation was performed in the absence of magnesium, nucleotides involved in hysteretic inhibition were not retained on the enzyme (Di Pietro et al., 1980) unless covalently bound by photoirradiation.

TDAB–Polyacrylamide Gel Electrophoresis. The eluate obtained after photoirradiation was concentrated about 4-fold by lyophilization for 1 h. The protein was depolymerized with 0.07 M TDAB and 5% β-mercaptoethanol in 50 mM potassium phosphate buffer, pH 4.0, for 2 h at room temperature. Heating of the samples was avoided to prevent a possible damage to the ester bond of NAP_3-ADP (Jeng & Guillory, 1977). Sucrose (1.7 mM) was added to increase the density of the samples as well as traces of bromophenol blue as tracking dye. The subunits of F_1-ATPase were separated by electrophoresis in 12% polyacrylamide gel in the presence of TDAB (Amory et al., 1980). This gel system was preferred to the sodium dodecyl sulfate–polyacrylamide gel system because it permitted a much better separation of the α- and β-subunits of the F_1-ATPase (Penin et al., 1984) in a shorter time (4 h at 50 mA). At this pH, it was checked that the ester bond of NAP_3-ADP was not hydrolyzed.

After electrophoresis, the gels corresponding to the photolabeled samples were cut into 1-mm slices. The slices were digested for 2 h in 1 mL of soluene 300 (Packard) at 50 °C. After these were cooled at room temperature, 10 mL of toluene containing 6 g of 2,5-diphenyloxazole and 75 mg of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene per L were added to each vial. The radioactivity was counted in a Beckman LS 7000 spectrometer.

Results

Hysteretic Inhibition of F_1-ATPase and Concomitant Nucleotide Binding Can Be Induced as Well by NAP_3-ADP in the Dark as by ADP. Figure 1 shows that preincubation of F_1-ATPase in the dark with NAP_3-ADP or ADP, in the

Table I: Influence of Preincubation of F_1-ATPase with ADP or with GMP-P(NH)P on [^{3}H]NAP_3-ADP Bound in the Dark to Enzyme[a]

expt	additions during preincubation	inhibition of ATPase activity (%)	bound [^{3}H]NAP_3-ADP (mol/mol of enzyme)
1	[^{3}H]NAP_3-ADP	74	2.5
2	ADP, [^{3}H]NAP_3-ADP	77	1.2
3	[^{3}H]NAP_3-ADP, GMP-P(NH)P	75	2.2

[a] F_1-ATPase (1 mg of protein/mL) was preincubated in the dark, at 30 °C, with 50 mM Tris-H_2SO_4, 1.5 mM $MgSO_4$, and 10% glycerol, pH 8.0. At t = 0, ADP (0.2 mM) was added to experiment 2; at t = 30 min, [^{3}H]NAP_3-ADP (0.3 mM, 48 mCi/mmol) was added to each asssay; at t = 45 min, GMP-P(NH)P (0.5 mM) was added to experiment 3. At t = 60 min, free nucleotides were removed by filtration–centrifugation through Sephadex G-50 (fine) equilibrated in the same medium in the absence of any nucleotide. The ATPase activity and the protein content were measured in the eluate and compared to the initial value [63 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$]. The bound [^{3}H]NAP_3-ADP was estimated by the radioactivity in the same eluate.

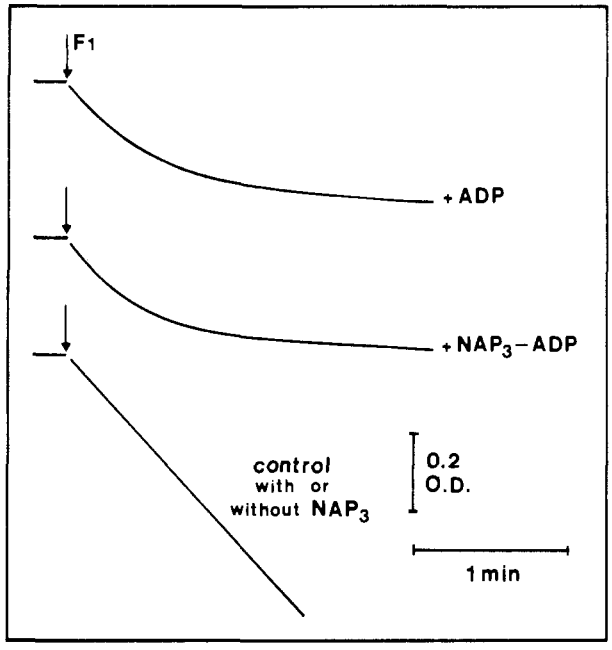

FIGURE 1: Recordings of the rate of ATP hydrolysis after preincubation of the mitochondrial F_1-ATPase in the presence or the absence of ADP, NAP_3-ADP, or NAP_3. F_1-ATPase (0.5 mg/mL) was preincubated, in the dark, without (control) or with 0.2 mM NAP_3 (control, lower curve), 0.2 mM NAP_3-ADP (middle curve), or 0.2 mM ADP (upper curve) as described under Experimental Procedures. After 30 min, a 1-μL-aliquot was transferred into the spectrophotometer cuvette to measure the ATPase activity.

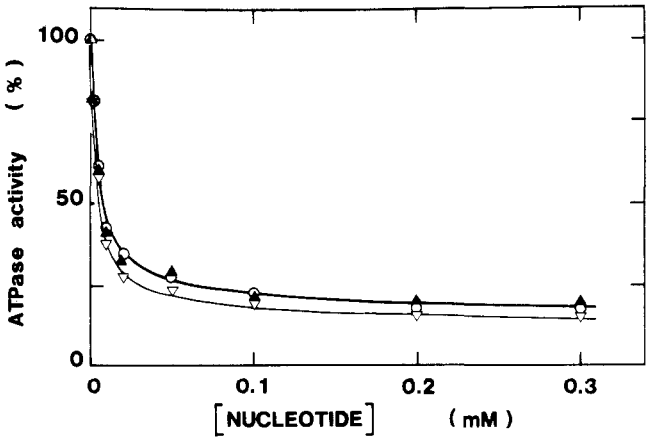

FIGURE 2: ADP- or NAP_3-ADP-induced inhibition of F_1-ATPase activity as a function of nucleotide concentration. F_1-ATPase was preincubated under the same conditions as those described in Figure 1 except that ADP (○) or NAP_3-ADP (▽, ▲) concentrations were varied in the preincubation medium as indicated in the figure. the activity was tested without photoirradiation (▲, ○) or after a 30-min photoirradiation (▽), as described under Experimental Procedures. One-hundred percent ATPase activity = 66 μmol of ATP min^{-1} (mg of protein)$^{-1}$.

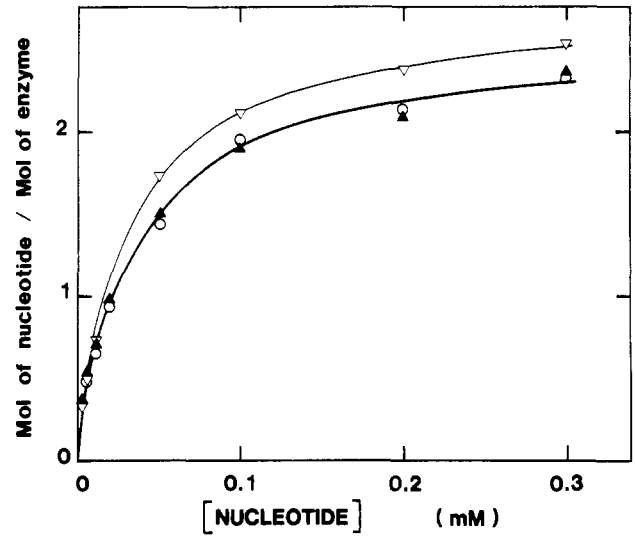

FIGURE 3: Variations of the amount of ^{3}H-labeled nucleotide remaining bound to the enzyme after gel filtration as a function of nucleotide concentration and of photoirradiation. F_1-ATPase was preincubated under the same conditions as those described in Figure 2. The radioactivity measured was that remaining bound after filtration–centrifugation through a Sephadex G-50 column equilibrated in the preincubation medium without added nucleotides. $MgSO_4$ (1.5 mM) was present in the Sephadex G-50 column except in the samples tested after photoirradiation (see Experimental Procedures). The bound [^{3}H]ADP or [^{3}H]NAP_3-ADP was estimated in a scintillation cocktail made of dioxane containing 50 g of naphthalene, 2.5 g of diphenyloxazole, and 100 mL of water per L. Symbols are as in Figure 2.

presence of $MgSO_4$, induced the same type of inhibition of the ATPase activity. The kinetics were biphasic: during the first seconds, the ATP hydrolysis started at a rate similar to that of the control but progressively diminished until a constant inhibited rate was reached. The preincubation of the enzyme with NAP_3 did not modify the control preincubated without any added nucleotide.

Figure 2 demonstrates that the inhibition increased in a similar manner with ADP or NAP_3-ADP concentration until a maximal inhibition of about 80% was reached at a concentration of about 50 μM. After photoirradiation of F_1-ATPase in the presence of NAP_3-ADP, which induced the covalent binding of the analogue, the percentage of inhibition slightly increased.

In Figure 3, the correlation between ADP binding and NAP_3-ADP binding is put forward. A maximal amount of 2.4 mol of ADP or NAP_3-ADP could be bound per mol of F_1-ATPase under the experimental conditions used. As in the case of the inhibition study, photoirradiation slightly increased the binding of NAP_3-ADP.

Reciprocal Prevention by ADP and NAP_3-ADP of Binding of Nucleotides Concomitant to Hysteretic Inhibition. Table

Table II: Influence of Preincubation of F_1-ATPase with NAP_3-ADP or with GMP-P(NH)P on Binding of [^{3}H]ADP Concomitant to Hysteretic Inhibition[a]

additions during preincubation	inhibition of ATPase activity (%) before photoirradiation	inhibition of ATPase activity (%) after photoirradiation	inhibition of ATPase activity (%) after [^{3}H]ADP addition	bound [^{3}H]ADP (mol/mol of enzyme)
none	0	3	85	2.45
NAP_3-ADP	78	87	88	1.45
GMP-P(NH)P	0	1.5	85	1.85
NAP_3-ADP, GMP-P(NH)P	78	88	88	1.05

[a] F_1-ATPase [1 mg protein/mL, 68 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$] was preincubated in the dark at 30 °C with 50 mM Tris–H_2SO_4, 1.5 mM $MgSO_4$, and 10% glycerol, pH 8.0, in the absence or in the presence of 0.5 mM NAP_3-ADP, 0.5 mM GMP-P(NH)P, or both for 30 min. The samples were photoirradiated for 45 min as described under Experimental Procedures and submitted to filtration–centrifugation through a Sephadex G-50 column equilibrated in the same medium but omitting $MgSO_4$. Then, [^{3}H]ADP (0.2 mM, 29 mCi/mmol) and 1.5 mM $MgSO_4$ were added to each sample. After 30 min, the bound [^{3}H]ADP, the ATPase activity, and the protein concentration were measured after a second filtration–centrifugation in the presence of $MgSO_4$, as in Table I.

I shows that preincubation of F_1-ATPase with ADP decreased the binding of [^{3}H]NAP_3-ADP from 2.5 to 1.2 mol/mol of F_1-ATPase. Besides, in experiment 2, the ADP-induced inhibition of the ATPase activity was not modified upon addition of [^{3}H]NAP_3-ADP (not shown). It was previously demonstrated that GMP-P(NH)P cannot occupy the site(s) responsible for ADP induced hysteretic inhibition (Baubichon et al., 1981), but it occupies catalytic sites since it is a competitive inhibitor of MgATP hydrolysis (Roux et al., 1984). The presence of GMP-P(NH)P in the preincubation medium slightly diminished the binding of [^{3}H]NAP_3-ADP from 2.5 to 2.2 mol/mol of F_1-ATPase. The same results were obtained whether GMP-P(NH)P was added before or after [^{3}H]-NAP_3-ADP.

The photoirradiation of F_1-ATPase with NAP_3-ADP decreased the binding of [^{3}H]ADP from 2.45 to 1.45 mol/mol of enzyme (Table II). Preincubation of F_1-ATPase with GMP-P(NH)P diminished the binding of [^{3}H]ADP from 2.45 to 1.85 mol/mol of enzyme. The photoirradiation of F_1-ATPase in the presence of both NAP_3-ADP and GMP-P(NH)P further decreased the binding of ADP to about 1 mol. Table II also shows that the photoirradiation procedure did not damage the enzyme whether GMP-P(NH)P was present or not. After photoirradiation of the enzyme preincubated with ADP in the presence or absence of GMP-P(NH)P, the hysteretic inhibition was not decreased. The photoirradiation increased from 78 to 87–88% the NAP_3-ADP-induced inhibition. An ADP addition did not increase the enzyme inhibition when NAP_3-ADP was already present.

Localization by Photolabeling of the Binding Site of [^{3}H]NAP_3-ADP Inducing Hysteretic Inhibition. In the following experiments, the purpose was to discriminate between unspecific binding of [^{3}H]NAP_3-ADP to subunits of F_1-ATPase and the specific binding correlated to the hysteretic inhibition, i.e., on the regulatory site. Two types of experiments were conducted in parallel.

In the first type of experiments, F_1-ATPase was preincubated in the presence of 0.2 mM ADP to prevent any binding of [^{3}H]NAP_3-ADP at the regulatory site. After 30 min, 0.5 mM GMP-P(NH)P in order to saturate catalytic sites and [^{3}H]-NAP_3-ADP at various concentrations (5–500 μM) in order to detect unspecific binding were added at the same time. In the second type of experiments, F_1-ATPase was incubated

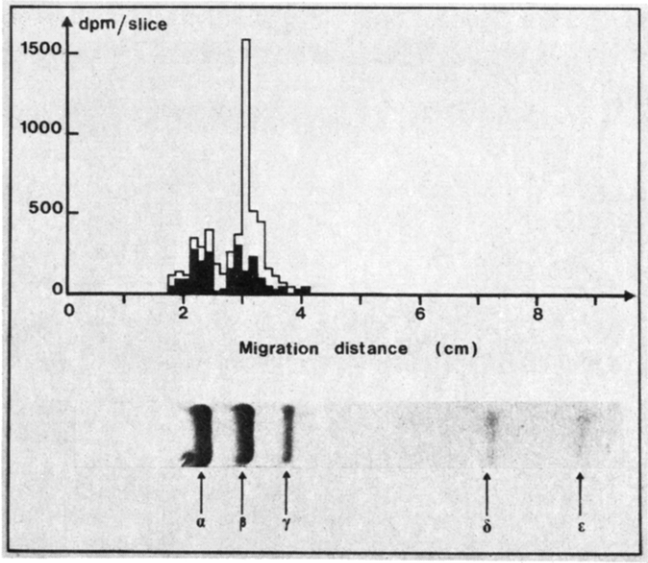

FIGURE 4: TDAB–polyacrylamide gel electrophoresis of F_1-ATPase photolabeled with 0.5 mM [^{3}H]NAP_3-ADP. The dark areas represent the labeling obtained when the enzyme was preincubated with ADP before [^{3}H]NAP_3-ADP addition. For other experimental conditions, see Experimental Procedures. The photoirradiation time was 30 min. A total of 25 μg of protein was subjected to gel electrophoresis.

Table III: Bound [^{3}H]NAP_3-ADP on α- and β-Subunits as a Function of Free NAP_3-ADP in the Presence and in the Absence of ADP[a]

free [^{3}H]NAP_3-ADP (μM)	dpm α +ADP	dpm α −ADP	dpm β +ADP	dpm β −ADP
5	74	126	286	457
25	400	543	400	1010
500	630	930	610	2680

[a] The experimental conditions are the same as those in Figure 4 except that [^{3}H]NAP_3-ADP concentration was varied. The specific radioactivity of [^{3}H]NAP_3-ADP was 80 dpm/pmol. After the radioactivity in the gel slices was measured, the counts corresponding to α- or β-subunits were added.

in the absence of ADP, and then the same assays were conducted to measure the total binding of [^{3}H]NAP_3-ADP in the presence of GMP-P(NH)P. The same concentrations of [^{3}H]NAP_3-ADP (5–500 μM) were used and produced both an increased binding and an increased inhibition as shown above in Figures 2 and 3. After photoirradiation, samples from all assays were analyzed by TDAB gel electrophoresis. The gels were sliced and counted. Figure 4 shows that β-subunits of F_1-ATPase are strongly labeled as compared to α-subunits, after photoirradiation in the presence of saturating concentrations of [^{3}H]NAP_3-ADP. Preincubation of F_1-ATPase with ADP prior to the addition of NAP_3-ADP essentially decreased the labeling of the β-subunit. GMP-P(NH)P used to occupy catalytic sites barely diminished the binding of NAP_3-ADP, as measured after filtration–centrifugation, and did not release the hysteretic inhibition (see above). Previous experiments (Roux et al., 1984) have shown that whether the enzyme was preincubated or not with ADP, GMP-P(NH)P behaved as a competitive inhibitor of MgATP. This means that GMP-P-(NH)P can occupy the hydrolytic site whether ADP is present or not at the regulatory site. It was checked here that the inhibition of the ATPase activity by NAP_3-ADP remained constant in the presence of GMP-P(NH)P, even after 2 h of incubation, time corresponding to the longest photoirradiation tested, which proves once more that GMP-P(NH)P cannot occupy the regulatory sites.

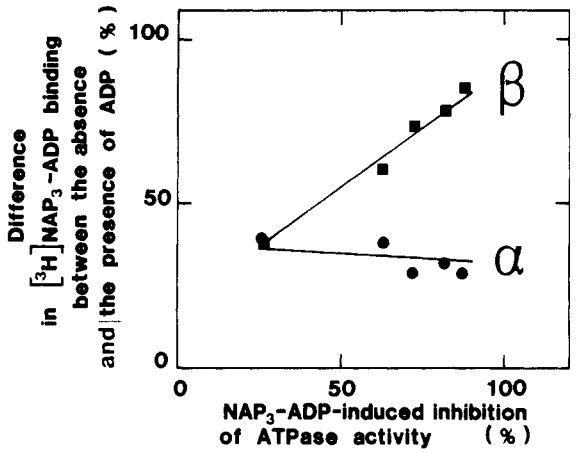

FIGURE 5: Correlation between the percentage of NAP_3-ADP-induced hysteretic inhibition of F_1-ATPase and the percentage of difference in [^{3}H]NAP_3-ADP binding in the absence or presence of ADP after photolabeling. The experimental conditions are the same as those in Figure 4 except that [^{3}H]NAP_3-ADP concentration was varied from 5 to 500 μM to obtain various extents of inhibition. For each experiment, the [^{3}H]NAP_3-ADP induced hysteretic inhibition was measured before photoirradiation in the assays that did not contain ADP. In each experiment, both samples (25 μg of protein) preincubated in the absence or in the presence of ADP were subjected to gel electrophoresis in parallel lanes. After the radioactivity in the gel slices was measured, the counts corresponding to the α- (●) or β-subunits (■) were added. The percentage of protection by ADP was calculated as follows: percentage for each subunit = [dpm_{-ADP} − dpm_{+ADP}]/[dpm_{-ADP}], where dpm_{-ADP} and dpm_{+ADP} respectively represent the assays preincubated in the absence and in the presence of ADP. Since four preparations of [^{3}H]NAP_3-ADP with different specific radioactivities (25, 37, 41, and 68 mCi/mmol) were used, the results are expressed as percent of the total binding.

Table III shows that when the NAP_3-ADP concentration varied from 5 to 500 μM, its binding increased on both α- and β-subunits in all assays. However, in the presence of ADP that prevents the binding of the analogue to the regulatory site, the labeling on α- and β-subunits is similar while, in the absence of ADP, the labeling is predominant on β-subunits. The difference (dpm in the absence of ADP minus dpm in the presence of ADP) can be used as an estimate of the binding of [^{3}H]NAP_3-ADP specific for hysteretic inhibition. This is further demonstrated in Figure 5 , where this difference increases only for the β-subunits as the inhibition produced by increasing concentrations of [^{3}H]NAP_3-ADP varied from 26 to 87%. On the contrary, no significant variation of the difference in counts was observed on α-subunits. Therefore, there is a direct correlation between the specific binding of [^{3}H]-NAP_3-ADP on β-subunits and the hysteretic inhibition, while no correlation appears in the case of α-subunits.

Discussion

The results described in this paper demonstrate that arylazido-β-alanyl-ADP (NAP_3-ADP) is an analogue very well suited for the study of the localization of the regulatory site of mitochondrial F_1-ATPase. Russell et al. (1976) proved that the ATP derivative (NAP_3-ATP) could act as a substrate when incubated with F_1-ATPase in the dark and serve as an affinity label after photoirradiation. Lunardi et al. (1981) have shown that the arylazido-β-aminobutyryl-ADP (NAP_4-ADP) binds to α- and β-subunits of *Escherichia coli* F_1-ATPase with a lesser affinity than ADP. The distribution of the bound photolabel between α and β in the absence of magnesium depended on its initial concentration.

In the present work, NAP_3-ADP behaved exactly as ADP to induce the hysteretic inhibition of pig heart F_1-ATPase, as previously defined (Di Pietro et al., 1980). The induced inhibition increased with NAP_3-ADP concentration in exactly the same range as ADP. The binding of [^{3}H]NAP_3-ADP in the dark was also identical with that of ADP. After photoirradiation, the binding of [^{3}H]NAP_3-ADP was slightly increased as well as the related inhibition. It is possible that this additional inhibition and binding are not related to the site responsible for the hysteretic inhibition and that NAP_3-ADP also binds to the catalytic sites.

The preincubation of F_1 with ADP prevents the binding of slightly more than 1 mol of NAP_3-ADP and vice versa. This mole of ADP or NAP_3-ADP should be relatively rapidly exchangeable under the experimental conditions used. On the contrary, during the same time, hysteretic inhibition would not be reversed, and the ADP bound at the regulatory site would not be exchanged since both processes have been shown to be slow (Di Pietro et al., 1980; Baubichon et al., 1981). This suggests, that, in experiment 2 of Table I, NAP_3-ADP is essentially bound at sites not specific for the hysteretic inhibition, that is, at catalytic or tightly-bound sites.

The total amount of labeled ADP or NAP_3-ADP bound to the enzyme did not represent only the binding of nucleotides at the site that is responsible for the hysteretic inhibition. Indeed, in assays with GMP-P(NH)P that did not prevent the hysteretic inhibition, the nucleoside diphosphate binding was decreased. It was shown previously that the guanosine analogues could not induce the hysteretic inhibition (Baubichon et al., 1981) while they could occupy the catalytic sites (Schuster et al., 1975; Roux et al., 1984). Therefore, GMP-P(NH)P was added in all further experiments aiming to localize the regulatory site in order to prevent as much as possible the binding of NAP_3-ADP at catalytic sites. Since, under these conditions, the extent of hysteretic inhibition did not decrease after photoirradiation, the migration of ADP or NAP_3-ADP from regulatory sites to the other noninhibitory sites is unlikely. Under these conditions, the linear correlation between the specific labeling by NAP_3-ADP of the β-subunit and the inhibition of ATPase activity strongly favors the location of the site responsible for the hysteretic inhibition on the β-subunit. Indeed, there is no such correlation for the labeling on the α-subunit. However, one could imagine that the photoactivable part of the molecule covalently binds to β even though the ADP part is causing inhibition by interacting with a site on a neighboring α. This hypothesis seems to be ruled out by the experiments of Lunardi et al. (1981) in which the length of the arm between the nucleotide and the azido group did not affect the relative binding on the α- and β-subunits of *E. coli* F_1-ATPase. The localization on the β-subunit of the regulatory site does not exclude that this site could be close to α in the enzyme.

By use of the analogue [*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), we have shown (Di Pietro et al., 1979, 1981) by kinetic experiments that there were two types of binding sites related to the inhibition of the ATPase activity. Esch & Allison (1979) have shown that 3 mol of FSBA was bound to F_1-ATPase, 1 mol to each β-subunit, while the binding to the α-subunits appeared nonspecific under their conditions. Their experiments did not permit the identification of the catalytic or regulatory role of these sites on the β-subunits. This study leads to a model in which only one nucleotide binding site on one β-subunit out of three would have a regulatory function responsible of the hysteretic inhibition. This conclusion is consistent with structural models proposed by several authors using quite different approaches. By X-ray diffraction studies, Amzel et al. (1982, 1983) suggested that "the three α- and β-subunits are not structurally equivalent

in the complex and that the α- and β-subunits which are structurally not equivalent may have different functional properties from the other α- and β-subunits". In addition, by modification of essential amino acid residues, Cross & Nalin (1982) have shown that reaction of F_1-ATPase with pyridoxal 5'-phosphate or phenylglyoxal resulted in the loss of one AMP-P(NH)P binding site while the modification of essential carboxyl residues or the binding of efrapeptin resulted in the loss of two sites. These differences in the reactivity to chemical reagents are consistent with a functional heterogeneity of the three α- or β-subunits and therefore with our model.

At a given time, the occupancy by ADP of the regulatory site on one β-subunit would decrease the ATP hydrolysis at the other sites. We have shown previously that the hydrolysis of many molecules of ATP at the catalytic sites does not chase labeled ADP bound at the regulatory site responsible for the hysteretic inhibition (Di Pietro et al., 1980). This excludes that all three sites on β-subunits become alternatively regulatory or catalytic as the F_1-ATPase turns over. But this does not rule out that the regulatory site occupied by ADP cannot be occupied by ATP at another given time under different conditions (for example, when F_1-ATPase is bound to the membrane) and can function as a catalytic site. It should also be recalled that the nucleotides bound at the regulatory site are different from the tightly bound nucleotides since the hysteretic inhibition and most of the concomitant ADP binding were released by ammonium sulfate precipitation (Di Pietro et al., 1980) while this treatment does not remove tightly bound nucleotides (Harris et al., 1978). In conclusion, there is a regulatory site on one β-subunit that is different from the catalytic and tightly bound nucleotide sites. The simplest explanation for our results would be that one of the three β-subunits contains one regulatory site while the two others would contain one catalytic site each. Cross et al. (1982), on the basis of kinetic experiments, suggested that there are three catalytic sites on beef heart F_1-ATPase. However, they could not rule out the possibility of the existence of a nucleotide binding site with a regulatory function in addition to two catalytic sites. Our results appear consistent with the latter alternative.

Acknowledgments

We thank F. Penin for the preparation of the F_1-ATPase and for his expert advice to perform the gel electrophoresis in the presence of TDAB. We are indebted to R. Lambrech for improving the English version of the manuscript and C. Van Herrewège for the art work.

References

Amory, A., Foury, F., & Goffeau, A. (1980) *J. Biol. Chem. 255*, 9353–9357.

Amzel, L. M., & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.

Amzel, L. M., Mc Kinney, M., Narayanan, P., & Pedersen, P. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5852–5856.

Baubichon, H., Godinot, C., Di Pietro, A., & Gautheron, D. C. (1981) *Biochem. Biophys. Res. Commun. 100*, 1032–1038.

Baubichon, H., Di Pietro, A., Godinot, C., & Gautheron, D. C. (1982) *FEBS Lett. 137*, 261–264.

Cross, R. L., & Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874–2881.

Cross, R. L., Grubmeyer, C., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12101–12105.

Di Pietro, A., Godinot, C., Bouillant, M. L., & Gautheron, D. C. (1975) *Biochimie 57*, 959–967.

Di Pietro, A., Godinot, C., Martin, J. C., & Gautheron, D. C. (1979) *Biochemistry 18*, 1738–1745.

Di Pietro, A., Penin, F., Godinot, C., & Gautheron, D. C. (1980) *Biochemistry 19*, 5671–5678.

Di Pietro, A., Godinot, C., & Gautheron, D. C. (1981) *Biochemistry 20*, 6312–6318.

Di Pietro, A., Godinot, C., & Gautheron, D. C. (1983) *Biochemistry 22*, 785–792.

Esch, F. S., & Allison, W. S., (1979) *J. Biol. Chem. 254*, 10740–10746.

Gautheron, D. C., Durand, R., Pialoux, N., & Gaudemer, Y. (1964) *Bull. Soc. Chim. Biol. 46*, 645–660.

Guillory, R. J., & Jeng, S. J. (1977) *Methods Enzymol. 46*, 259–288.

Harris, D. A., Gomez-Fernandez, J. C., Klungsoyr, L., & Radda, G. K. (1978) *Biochim. Biophys. Acta 504*, 364–383.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lunardi, J., Lauquin, G. J. M., & Vignais, P. V. (1981) *Biochemistry 20*, 473–480.

Pedersen, P. L. (1975) *Biochem. Biophys. Res. Commun. 64*, 610–616.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Penin, F., Godinot, C., & Gautheron, D. C. (1979) *Biochim. Biophys. Acta 548*, 63–71.

Penin, F., Godinot, C., & Gautheron, D. C. (1984) *Biochim. Biophys. Acta 775*, 239–245.

Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322–3329.

Roux, B., Fellous, G., & Godinot, C. (1984) *Biochemistry 23*, 534–537.

Russell, J., Jeng, S. J., & Guillory, R. J. (1976) *Biochem. Biophys. Res. Commun. 70*, 1225–1234.

Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 7848–7853.