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# STUDIES OF THE NUCLEOTIDE-BINDING SITES ON THE MITOCHONDRIAL $F_1$-ATPase THROUGH THE USE OF A PHOTOACTIVABLE DERIVATIVE OF ADENYLYL IMIDODIPHOSPHATE

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**(1) *N*-4-Azido-2-nitrophenyl-γ-[$^3$H]aminobutyryl-Ado*PP*[NH]*P* ($NAP_4$-Ado*PP*[NH]*P*) a photoactivable derivative of 5-adenylyl imidodiphosphate (Ado*PP*[NH]*P*), was synthesized. (2) Binding of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* to soluble ATPase from beef heart mitochrondria ($F_1$) was studied in the absence of photoirradiation, and compared to that of [$^3$H]Ado*PP*[NH]*P*. The photoactivable derivative of Ado*PP*[NH]*P* was found to bind to $F_1$ with high affinity, like Ado*PP*[NH]*P*. Once [$^3$H]$NAP_4$-Ado*PP*[NH]*P* had bound to $F_1$ in the dark, it could be released by Ado*PP*[NH]*P*, ADP and ATP, but not at all by $NAP_4$ or AMP. Furthermore, preincubation of $F_1$ with unlabeled Ado*PP*[NH]*P*, ADP, or ATP prevented the covalent labeling of the enzyme by [$^3$H]$NAP_4$-Ado*PP*[NH]*P* upon photoirradiation. (3) Photoirradiation of $F_1$ by [$^3$H]$NAP_4$-Ado*PP*[NH]*P* resulted in covalent photolabeling and concomitant inactivation of the enzyme. Full inactivation corresponded to the binding of about 2 mol [$^3$H]$NAP_4$-Ado*PP*[NH]*P*/mol $F_1$. Photolabeling by $NAP_4$-Ado*PP*[NH]*P* was much more efficient in the presence than in the absence of $MgCl_2$. (4) Bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was localized on the α- and β-subunits of $F_1$. At low concentrations (less than 10 μM), bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was predominantly localized on the α-subunit; at concentrations equal to, or greater than 75 μM, both α- and β-subunits were equally labeled. (5) The extent of inactivation was independent of the nature of the photolabeled subunit (α or β), suggesting that each of the two subunits, α and β, is required for the activity of $F_1$. (6) The covalently photolabeled $F_1$ was able to form a complex with aurovertin, as does native $F_1$. The ADP-induced fluorescence enhancement was more severely inhibited than the fluorescence quenching caused by ATP. The percentage of inactivation of $F_1$ was virtually the same as the percentage of inhibition of the ATP-induced fluorescence quenching, suggesting that fluorescence quenching is related to the binding of ATP to the catalytic site of $F_1$.**

## Introduction

Ado*PP*[NH]*P* is a potent competitive, nonhydrolyzable, inhibitor of both membrane-bound and solubilized mitochondrial $F_1$-ATPase [1–4]. It does not inhibit ATP synthesis in submitochondrial particles, a finding that was at first interpreted as evidence of separate sites for hydrolysis and synthesis of ATP [2,5]. Ado*PP*[NH]*P* is able to complete with ADP for binding to isolated $F_1$. However, a large excess of Ado*PP*[NH]*P* does not completely displace bound ADP; this observation led Penefsky [2] to suggest that the bound ADP which resisted removal could be attached to a site

Abbreviations: $F_1$, soluble mitochondrial ATPase from beef heart mitochondria; Ado*PP*[NH]*P*, 5-adenylyl imidodiphosphate; $NAP_4$, *N*-4-azido-2-nitrophenyl-γ-aminobutyric acid; $NAP_4$-Ado*PP*[NH]*P*, *N*-4-azido-2-nitrophenyl-γ-aminobutyryl-Ado*PP*[NH]*P*; $NAP_4$-ADP, *N*-4-azido-2-nitrophenyl-γ-aminobutyryl-ADP.

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specific for ATP synthesis. Since Ado*PP*[NH]*P* binds tenaciously to $F_1$, it has been used to determine the total amount of nucleotide-binding sites in this enzyme. Preparations of $F_1$ which contain 3 mol of tightly bound ADP plus ATP per mol of $F_1$ still have two additional sites which may be engaged in reversible binding of Ado*PP*[NH]*P* [6] or ADP [7], indicating that there are at least five available nucleotide-binding sites in $F_1$. This is corroborated by the fact that up to five sites can be titrated by Ado*PP*[NH]*P* in nucleotide-depleted $F_1$ [6]. Thus, Ado*PP*[NH]*P* appears to substitute for ADP (or ATP), to bind to any ADP/ATP site in $F_1$. Assuming that nucleotide-binding sites in $F_1$ consist of both catalytic and regulatory sites, Schuster et al. [8] concluded, on the basis of kinetic data, that Ado*PP*[NH]*P* binds preferentially to the regulatory sites. It has been suggested that the regulatory sites are located on the $\alpha$-subunits [9]. Although the above data make it clear that Ado*PP*[NH]*P* is an interesting probe of nucleotide sites in $F_1$, no evidence has yet been presented concerning the localization and the affinity of Ado*PP*[NH]*P* for the different subunits of $F_1$. The purpose of the present paper is precisely an attempt to localize the Ado*PP*[NH]*P*-binding sites on $F_1$ through the use of a photolabeled derivative of Ado*PP*[NH]*P*. In these studies we also investigated the fluorescence response of the photolabeled aurovertin-$F_1$ complex to the binding of ADP and ATP.

## Materials and Methods

### *Materials*

4-[2,3-$^3$H]Aminobutyric acid (45 Ci/mmol) was obtained from the Commissariat à l'Energie Atomique (Centre d'Etudes Nucléaires, Saclay, France). [2,8-$^3$H]Ado*PP*[NH]*P* (17 Ci/mmol) was purchased from ICN Pharmaceuticals Inc. (U.S.A.). As Ado*PP*[NH]*P* preparations are often contaminated by ATP which accumulates during storage [10], the samples used in this work were routinely checked for purity by chromatographic analysis of the nucleotides [11], or by the luciferine-luciferase assay [12]; the ATP found was less than 0.15% of the total Ado*PP*[NH]*P*, and therefore could not be considered as a perturbing contaminant in the experiments described thereafter. Synthesis of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was performed by the same method as that described by Guillory and Jeng [13] for the synthesis of $NAP_4$-ATP. The labeling was on 4-[2,3$^3$H]aminobutyric acid [14] and not on Ado*PP*[NH]*P*. Introduction of $^3$H into $NAP_4$ rather than into Ado*PP*[NH]*P* was dictated by the fact that during photolabeling and subsequent operations like SDS-polyacrylamide gel electrophoresis, the Ado*PP*[NH]*P* moiety of $NAP_4$-Ado*PP*[NH]*P* can be damaged, but not the $NAP_4$ portion of the molecule. [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was purified by cellulose thin-layer chromatography (Schleicher and Schüll, F1440) in 1-butanol/water/acetic acid (5:3:2, v/v); its $R_f$ value was 0.52. That the product of synthesis was the monophotoactivable derivative of Ado*PP*[NH]*P* was established by using [$^3$H]$NAP_4$ of known specific radioactivity. Aurovertin D was purified from *Calcarisporium arbuscula* NRRL 3705 [15], and stored as an ethanolic solution protected from light. Aurovertin fluorescence was measured at 30°C in a Perkin Elmer MPF 2A fluorimeter, the excitation wavelength being set at 365 nm and the emission wavelength at 470 nm. The molar extinction coefficient of the aurovertin preparation used in this work was 35100 at 368 nm.

### *Biological assays*

$F_1$ was prepared from beef heart mitochrondria according to the method of Knowles and Penefsky [16], and stored at 4°C as an ammonium sulfate precipitate. Before use, the suspension was centrifuged and the pellet solubilized in a buffered medium containing 50 mM Tris-HCl and either EDTA or $MgCl_2$, as indicated in the text (final pH 7.5). The $F_1$-ATPase solution was finally freed from ammonium sulfate by filtration through Sephadex G-50 (fine) (Pharmacia Chemicals) equilibrated with the same buffer [17]. A molecular weight of 360000 for $F_1$ was used for calculations [18].

*Assay of ATPase activity.* ATPase activity of $F_1$ was routinely assayed at 30°C with an ATP-regenerating system. ATP hydrolysis was started by addition of an aliquot of $F_1$ (1–2 $\mu$g) to a medium containing 4 mM phospho*enol*pyruvate, 20 $\mu$g pyruvate kinase, 10 mM ATP, 5 mM $MgCl_2$ and 40 mM Tris-HCl, pH 8.0, in a final volume of 0.5

ml. The excess of ATP with respect to $Mg^{2+}$ used in this assay is in fact required for optimal activity of $F_1$ [19]. After a 5 min incubation, the reaction was terminated by addition of 0.2 ml trichloroacetic acid (50%, w/v). The phosphate released from ATP by hydrolysis was estimated by the method of Fiske and SubbaRow [20]. Protein concentration was measured as reported by Bradford [21] with bovine serum albumin as standard.

*Reversible binding of [$^3$H]NAP$_4$-AdoPP[NH]P to $F_1$ in the dark*

Samples of $F_1$ were incubated in the dark as detailed in the text. After incubation with [$^3$H]NAP$_4$-Ado*PP*[NH]*P* for 60 min at 25°C, free nucleotides were separated from bound nucleotides by the elution-centrifugation method of Penefsky [17], using a 1 ml syringe equipped with a porous polyethylene disk and filled with 1 ml Sephadex G-50 (fine) equilibrated with 50 mM Tris-HCl and EDTA or $MgCl_2$, final pH 7.5. The bound radioactivity and the protein concentration of the filtrates were determined.

*Photolabeling assays.* Photolabeling was carried out under conditions similar to those previously described [22]. In brief, $F_1$ was preincubated in the dark for 15 min at 25°C in a medium containing 50 mM Tris-HCl and 2 mM EDTA or 3 mM $MgCl_2$, final pH 7.5, in the presence of [$^3$H]NAP$_4$-Ado*PP*[NH]*P* in 5-ml glass tubes. The tubes exposed to light irradiation (Sylvania ELC lamp, 250 W) at a distance of 10 cm from the light source were rotated horizontally at 200 rpm for 20 min in a thermostatically controlled bath at 20°C. Following photoirradiation, the samples were supplemented with 5 mM Ado*PP*[NH]*P* to displace the noncovalently bound photolabel, and the bound and free [$^3$H]NAP$_4$-Ado*PP*[NH]*P* were separated by the elution-centrifugation method of Penefsky [17] using short Sephadex G-50 (fine) columns.

*Gel electrophoresis.* SDS-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [23], using 12% acrylamide gel, a migration period of 16 h and a current of 5 mA/gel. After staining and destaining, the gels were scanned and sliced. Each slice (1 mm) was digested overnight with 1 ml of 15% $H_2O_2$ at 60°C, and radioactivity was measured by liquid scintillation counting, using the scintillant of Patterson and Greene [24].

*Adenine nucleotide determination*

Adenine nucleotides were extracted from $F_1$ by perchloric acid (final concentration 6.6%) for 30 min at 0°C. After centrifugation, the supernatant was neutralized with KOH and the perchlorate precipitate eliminated. Adenine nucleotides were determined using a luciferin-luciferase method as described by Lundin et al. [12].

## Results

*Binding of [$^3$H]NAP$_4$-AdoPP[NH]P to $F_1$ in the dark*

The $F_1$ preparations used in the present experiments were not depleted of ADP and ATP. Assays made on two different batches of $F_1$ gave an average of 1.7 mol ADP and 1.1 mol ATP tightly bound per mol $F_1$. This nondepleted $F_1$ could still be susceptible to bind [$^3$H]Ado*PP*[NH]*P*. Addition of [$^3$H]Ado*PP*[NH]*P* at low (12 $\mu$M) and high (120 $\mu$M) concentrations resulted in the binding of 1.5 and 3.4 mol [$^3$H]Ado*PP*[NH]*P*/mol $F_1$, respectively (Table I). When [$^3$H]Ado*PP*[NH]*P* was used at the highest concentration, 120 $\mu$M, its binding was concomitant with the release of a small amount of tightly bound ADP, suggesting that possibly a fraction of the tightly bound ADP can be replaced by Ado*PP*[NH]*P*. However, there was a net increase in the amount of total bound nucleotides that amounted to up to 5.1 mol/mol $F_1$.

Titrations of Ado*PP*[NH]*P*-binding sites was previously reported by Garrett and Penefsky [6]. The data showed that in a $MgCl_2$-supplemented medium, two equivalent binding sites could be filled with [$^3$H]Ado*PP*[NH]*P* with a $K_d$ value of 1.3 $\mu$M. Titration of $F_1$ by [$^3$H]NAP$_4$-Ado*PP*[NH]*P* in the dark is illustrated in Fig. 1. The binding affinity and the plateau of saturation depended on the presence of $MgCl_2$. In the $MgCl_2$-supplemented medium, the number of saturable sites was slightly above 2 mol/mol $F_1$. The Scatchard plot of the binding data showed some heterogeneity; however, a large percentage of sites (over 70%) appeared to be homogeneous, with an approximate $K_d$ value of 3 $\mu$M. These values

TABLE I

ADENINE NUCLEOTIDE CONTENT OF BEEF HEART MITOCHONDRIAL $F_1$, AND COMPLEMENTARY BINDING OF [$^3$H]Ado*PP*[NH]*P*

Control $F_1$ was subjected, prior to adenine nucleotide determination to two precipitations by ammonium sulfate and to a passage on a Sephadex G-50 column. For [$^3$H]Ado*PP*[NH]*P* binding, the $F_1$ preparation after ammonium sulfate precipitation was incubated for 60 min with [$^3$H]Ado*PP*[NH]*P* at 25°C, in the presence of 50 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$, and then filtered on Sephadex G-50. After gel filtration, the $F_1$ was extracted by perchloric acid, and ADP and ATP were assayed in the neutralized perchloric extracts (cf. Methods). Bound [$^3$H]Ado*PP*[NH]*P* was calculated from the $^3$H radioactivity in the perchloric acid extract of $F_1$. The $M_r$ used for $F_1$ was 360000 [18]. Results are expressed as mol/mol $F_1$.

| Conditions | Bound ADP | Bound ATP | Bound [$^3$H]Ado*PP*[NH]*P* | Total amount of bound adenine nucleotides |
|---|---|---|---|---|
| $F_1$ (control) | 1.7 | 1.1 | – | 2.8 |
| $F_1$ + 12 μM [$^3$H]Ado*PP*[NH]*P* | 1.6 | 1.1 | 1.5 | 4.2 |
| $F_1$ + 120 μM [$^3$H]Ado*PP*[NH]*P* | 0.8 | 0.9 | 3.4 | 5.1 |

are quite close to those found by Garrett and Penefsky [6] for the binding of Ado*PP*[NH]*P* to $F_1$ (see above), indicating that the affinities of Ado*PP*[NH]*P* and the corresponding photolabel for $F_1$ are in the same range. A similar binding assay carried with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* in the EDTA-supplemented medium revealed a single binding site with low affinity ($K_d$ = 40 μM). Thus, the effect of $MgCl_2$ is particularly striking on the binding affinity of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* to $F_1$.

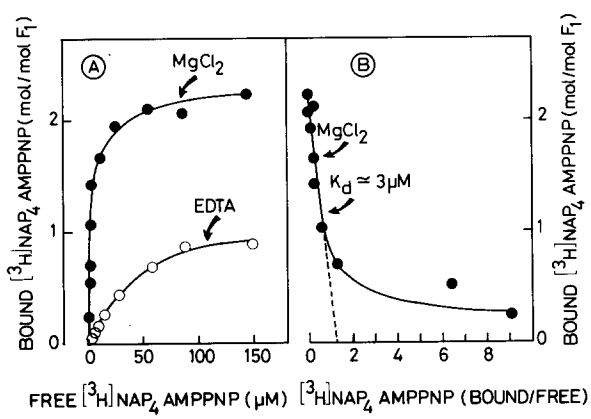


Fig. 1. Titration of $F_1$ with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* in the dark. $F_1$ (30 μg protein) was incubated in a series of tubes with 0.1 ml of medium made of 50 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$ (or 1 mM EDTA) in the presence of increasing concentrations of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* up to 150 μM. After equilibration (60 min at 25°C), bound and free [$^3$H]$NAP_4$-Ado*PP*[NH]*P* were separated by filtration on Sephadex G-50 as described in Methods. (A) Direct plot of bound ligand against free ligand in a $MgCl_2$ or EDTA medium. (B) Scatchard plot of the binding data ($MgCl_2$ medium). Note the curvilinearity of the plot at very low concentrations of the photolabel, which suggests transition from a very high affinity ($K_d$ < 50 nM) to moderate affinity binding ($K_d$ = 3 μM). AMPPNP, Ado*PP*[NH]*P*.

Kinetics of binding of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* in the dark, carried out first in an EDTA medium, and then in a $MgCl_2$ medium, are shown in Fig. 2. In accordance with titration data of Fig. 1, addition of $MgCl_2$ virtually doubled the total number of binding sites. [$^3$H]$NAP_4$-Ado*PP*[NH]*P* bound in the dark was slowly released upon addition of ADP. Similar experiments carried out with [$^3$H]Ado*PP*[NH]*P* led to similar results. The rates of release of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* bound in the dark upon addition of various adenine nucleotides and $NAP_4$ are compared in Fig. 3. Bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was released by Ado*PP*[NH]*P*, ADP and ATP virtually to the same extent, but not at all by $NAP_4$ or AMP. These data hold for binding assays in an $MgCl_2$ medium (Fig. 3) or in an EDTA medium (not shown). It may be noted that the rate of release induced by Ado*PP*[NH]*P*, ADP or ATP slows down after a few minutes. This was also observed for displacement of bound [$^3$H]Ado*PP*[NH]*P* by Ado*PP*[NH]*P*, ADP or ATP (not shown), and could be explained by some conformational change of the binding site after Ado*PP*[NH]*P* interaction. This conformational change takes time; the longer the period of incubation of $F_1$ with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* or [$^3$H]Ado*PP*[NH]*P*, the slower

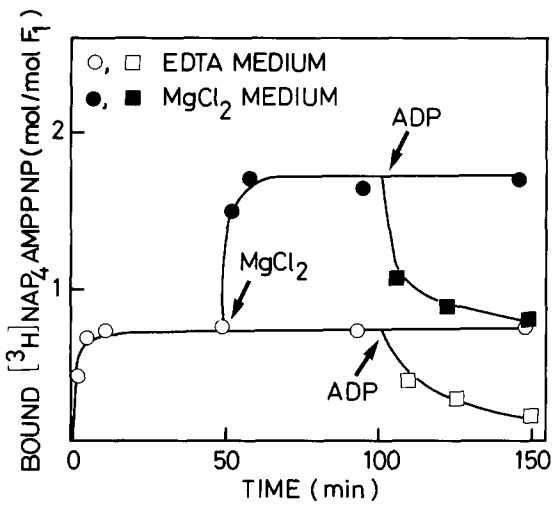


Fig. 2. Effect of EDTA and $MgCl_2$ on the binding of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* to $F_1$ in the dark. $F_1$ (42 μg protein) was incubated at 25°C in a series of tubes in 0.1 ml of a medium made of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, and 70 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. After the bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* had reached a plateau, 5 mM $MgCl_2$ was added. Later (100 min), 2 mM ADP was added. Bound and free [$^3$H]$NAP_4$-Ado*PP*[NH]*P* were separated by Sephadex G-50 filtration (cf. Methods). Additions of $MgCl_2$ and ADP are represented by arrows. AMPPNP, Ado*PP*[NH]*P*.

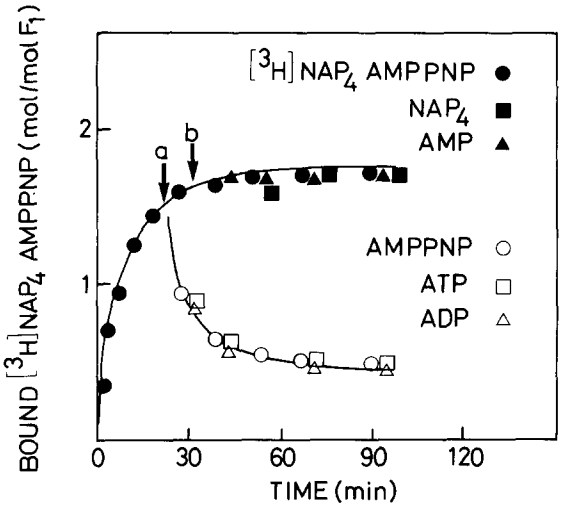


Fig. 3. Effect of Ado*PP*[NH]*P*, ADP, ATP, $NAP_4$ and AMP on the binding of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* to $F_1$ in the dark. $F_1$ (40 μg protein) was incubated in 50 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$ in the presence of 50 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. At the time indicated by arrow a, 3 mM Ado*PP*[NH]*P*, 3 mM ADP or 3 mM ATP was added. When ATP was used, ADP was generated by hydrolysis; however, the release of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was very fast and most likely due to the added ATP rather than to the newly generated ADP. Arrow b corresponds to addition of 3 mM AMP or 1 mM $NAP_4$. Bound and free [$^3$H]$NAP_4$-Ado*PP*[NH]*P* were separated by Sephadex G-50 filtration (cf. Methods). AMPPNP, Ado*PP*[NH]*P*.

the release of the bound [$^3$H]nucleotide by Ado*PP*[NH]*P*, ADP or ATP.

*Covalent photolabeling of $F_1$ by [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. Specificity of the labeling and concomitant inactivation of $F_1$*

Photolabeling of $F_1$ by $NAP_4$-Ado*PP*[NH]*P* was accompanied by inactivation of the ATPase activity (Fig. 4). Half inactivation corresponded to the binding of 1 mol [$^3$H]$NAP_4$-Ado*PP*[NH]*P*/mol $F_1$. Extrapolation to full inhibition corresponded to the binding of 2 mol [$^3$H]$NAP_4$-Ado*PP*[NH]*P*/mol $F_1$.

The labeling specificity with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* was confirmed by the competitive effect of nucleotides added to $F_1$ during the preincubation period in the dark, prior to photoirradiation (Table II). Preincubation with an excess of unlabeled Ado*PP*[NH]*P*, ADP, or ATP substantially decreased the amount of bound radioactivity; this indicated that the $NAP_4$ moiety was not

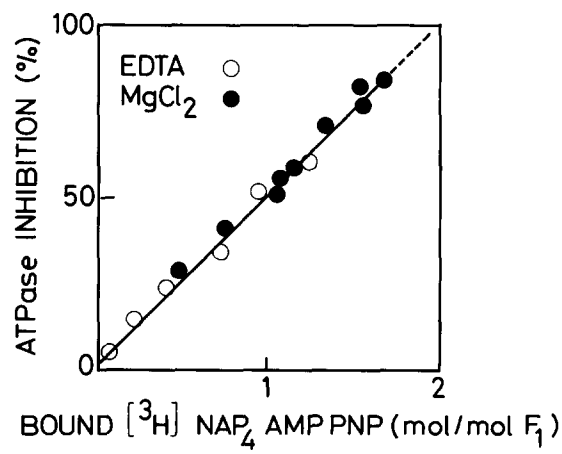


Fig. 4. Correlation between covalent photolabeling and photoinactivation of $F_1$ by [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. $F_1$ (60 μg protein) in 0.1 ml of a 50 mM Tris-HCl buffer, pH 7.5, supplemented with either 2 mM EDTA or 3 mM $MgCl_2$ was photoirradiated in the presence of varying concentrations (up to 100 μM) of [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. After Sephadex G-50 filtration, the bound radioactivity, the protein content and the ATPase activity of the filtrate were determined. AMPPNP, Ado*PP*[NH]*P*.

TABLE II

COVALENT PHOTOLABELING OF [$^3$H]$NAP_4$-Ado*PP*[NH]*P* TO $F_1$. COMPETITIVE EFFECT OF NUCLEOTIDES ADDED PRIOR TO PHOTOIRRADIATION

$F_1$ (50 μg protein) in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.5, supplemented with 2 mM EDTA or 3 mM $MgCl_2$, was photoirradiated in the presence of 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. Where indicated, Ado*PP*[NH]*P*, ADP, ATP and AMP were added with the photolabel prior to photoirradiation, as described in Methods. The bound [$^3$H]radioactivity was determined after Sephadex G-50 filtration (cf. Methods). Since ATP in the presence of $MgCl_2$ was hydrolyzed to give ADP and $P_i$, this assay was omitted (row 4).

| Additions | Bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* (mol/mol $F_1$) | |
|---|---|---|
| | $MgCl_2$ medium | EDTA medium |
| 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 1.57 | 1.00 |
| 1 mM Ado*PP*[NH]*P* plus 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 0.11 | 0.09 |
| 2 mM ADP plus 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 0.17 | 0.13 |
| 2 mM ATP plus 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | – | 0.11 |
| 2 mM AMP plus 25 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 1.53 | 1.06 |

significantly involved in binding. On the other hand, AMP which is not a substrate for $F_1$ was without effect. Thus, $NAP_4$-Ado*PP*[NH]*P* is specifically recognized by $F_1$. The two main criteria, affinity (see above section) and specificity, that are the basis for appropriate use of any photolabel are therefore met in the case of $NAP_4$-Ado*PP*[NH]*P* and $F_1$.

Another experiment was carried out with a partially photoinactivated $F_1$ to check whether the sites that had not been photolabeled by [$^3$H]$NAP_4$-Ado*PP*[NH]*P* were still able to react with, and bind the original ligand [$^3$H]Ado*PP*[NH]*P*. The $F_1$ preparation in the experiment of Fig. 5 was photoinactivated to about 75% and photolabeled to an extent of 1.6 mol [$^3$H]$NAP_4$-Ado*PP*[NH]*P*/mol $F_1$. To samples of this photolabeled $F_1$, [$^3$H]Ado*PP*[NH]*P* was added at increasing concentrations and allowed to equilibrate. A clear saturable binding for [$^3$H]-Ado*PP*[NH]*P* was observed with a plateau corresponding to a total amount of [$^3$H]Ado*PP*[NH]*P* and [$^3$H]$NAP_4$-Ado*PP*[NH]*P* of 2.4 mol/mol $F_1$.

One might argue that the $NAP_4$ moiety of the photoactivable Ado*PP*[NH]*P* may induce binding artifacts. We previously showed [14] that the $NAP_4$ moiety of $NAP_4$-ADP had the capability of binding to $F_1$, and that this binding was readily displaced by uncouplers; however, the affinity of $NAP_4$ for $F_1$ was quite low; for example, using a concentration of [$^3$H]$NAP_4$ as high as 40 μM, the amount of bound [$^3$H]$NAP_4$ per mol of $F_1$ was

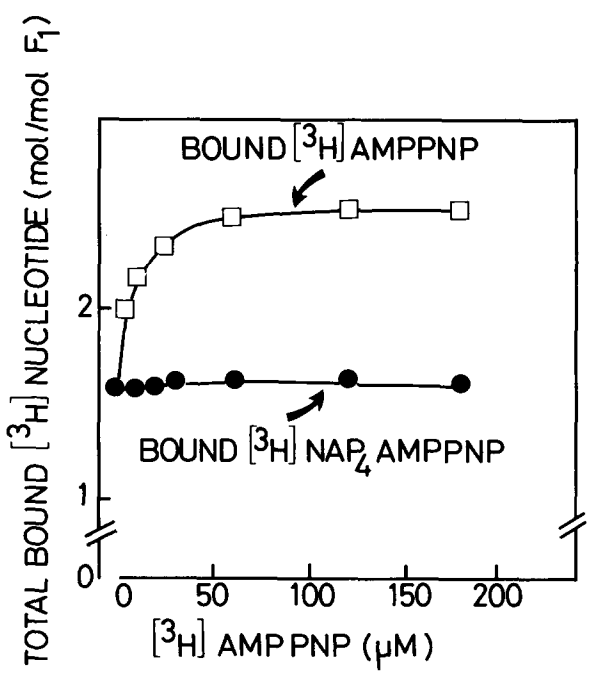


Fig. 5. Binding of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* to a partially photoinactivated $F_1$ by photoirradiation with [$^3$H]$NAP_4$-Ado*PP*[NH]*P*. A sample of $F_1$ was photoinactivated to about 75% by photoirradiation with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* (1.6 mol covalently bound photolabel/mol $F_1$). The nucleotide sites still available were titrated with [$^3$H]$NAP_4$-Ado*PP*[NH]*P* under the same conditions as in Fig. 1. AMPPNP, Ado*PP*[NH]*P*.

only 0.10 mol. Interference of $NAP_4$ with specific binding of $NAP_4$-Ado*PP*[NH]*P* to $F_1$ can therefore be considered of minor importance.

*Localization of bound [$^3$H]$NAP_4$-AdoPP[NH]P on $F_1$ subunits. Effect of the concentration of [$^3$H]$NAP_4$-AdoPP[NH]P on photolabeling of α- and β-subunits*

The distribution pattern of the covalently bound [$^3$H]$NAP_4$-Ado*PP*[NH]*P* between the $F_1$ subunits was determined after SDS-polyacrylamide gel electrophoresis of photolabeled $F_1$. The radioactivity was located essentially in the α- and β-subunits as previously found after photolabeling mitochondrial and bacterial ATPases by $NAP_4$-ADP [22,25].

Varying the concentration of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* in the photolabeling medium markedly modified the distribution of the photolabel between the α- and β-subunits (Table III). At low concentrations, [$^3$H]$NAP_4$-Ado*PP*[NH]*P* did mainly bind to the α-subunits. For example, using 7 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* in the presence of $MgCl_2$ (row 3, Table III), the amount of bound photolabel was 0.70 mol/mol $F_1$ (corresponding to about 35% inactivation), and 90% of the bound photolable was on the α-subunit, the remainder being on the β-subunit. At a concentration 10-times higher (75 μM) resulting in the binding of 1.45 mol of bound photolabel per mol of $F_1$, the photolabel was nearly equally distributed between the α- and β-subunits (row 6, Table III).

*Effect of ATP and ADP on the fluorescence of the photolabeled $F_1$-aurovertin complex*

It is well known that the aurovertin fluorescence is increased when aurovertin binds to $F_1$, and that the fluorescence of the $F_1$-aurovertin complex is enhanced by ADP and quenched by ATP [25,26]. The fluorescence enhancement due to binding of aurovertin to $F_1$ was the same whether or not $F_1$ was covalently photolabeled by $NAP_4$-Ado*PP*[NH]*P* provided that the extent of photoinactivation was less than 30–40%. When $F_1$ was extensively photoinactivated, the fluorescence response of aurovertin was somewhat diminished; for example, with a sample of $F_1$ photoinactivated

TABLE III

EFFECT OF INCREASING CONCENTRATIONS OF [$^3$H]$NAP_4$-Ado*PP*[NH]*P* ON COVALENT PHOTOLABELING OF α- AND β-SUBUNITS OF $F_1$

$F_1$ (40 μg protein) was photoirradiated in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.5, supplemented with either 2 mM EDTA or 3 mM $MgCl_2$ in the presence of the indicated concentrations of [$^3$H]$NAP_4$-Ado*PP*[NH]*P* as described in Methods. The photoirradiated samples were then subjected to SDS-polyacrylamide gel electrophoresis. After staining, the gels were sliced as described in Methods, and the radioactivity bound to the α- and β-subunits determined.

| Conditions | Covalently bound | % of bound $^3$H radioactivity | |
|---|---|---|---|
| | [$^3$H]$NAP_4$-Ado*PP*[Nh]*P* | α-subunit | β-subunit |
| $MgCl_2$ medium | | | |
| 1 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 0.32 | 95 | 5 |
| 3 μM | 0.36 | 90 | 10 |
| 7 μM | 0.70 | 90 | 10 |
| 22 μM | 0.86 | 82 | 18 |
| 45 μM | 1.32 | 70 | 30 |
| 75 μM | 1.45 | 60 | 40 |
| EDTA medium | | | |
| 1 μM [$^3$H]$NAP_4$-Ado*PP*[NH]*P* | 0.09 | 95 | 5 |
| 5 μM | 0.25 | 80 | 20 |
| 15 μM | 0.52 | 79 | 21 |
| 30 μM | 0.65 | 68 | 32 |
| 75 μM | 0.75 | 65 | 35 |
| 112 μM | 0.91 | 63 | 37 |

TABLE IV

EFFECT OF PHOTOLABELING OF $F_1$ WITH $NAP_4$-Ado*PP*[NH]*P* ON THE FLUORESCENCE OF BOUND AUROVERTIN. CORRELATION BETWEEN THE STIMULATORY EFFECT OF ADP AND THE QUENCHING EFFECT OF ATP ON FLUORESCENCE OF THE AUROVERTIN PHOTOLABELED $F_1$ COMPLEX, AND THE INACTIVATION OF PHOTOLABELED $F_1$

The sample of photolabeled $F_1$ used had been photoirradiated in the presence of 46 μM $NAP_4$-Ado*PP*[NH]*P* in an $MgCl_2$ medium, and photoinactivated to different extents. The photolabeled $F_1$ was recovered free of the photoproducts by filtration on Sephadex G-50. The fluorescence assay was carried out on 0.15 mg of control $F_1$ or $NAP_4$-Ado*PP*[NH]*P*-photolabeled $F_1$ in 2 ml of 0.2 M sucrose, 30 mM Tris-acetate, pH 7.5. Aurovertin was added to a final concentration of 1 μM, ADP to 0.1 mM and ATP to 3 mM. The ATPase activity was determined on aliquots of control and photolabeled $F_1$ (cf. Methods).

| Conditions of preincubation | ADP-induced increase in fluorescence (%) | ATP-induced quenching of fluorescence (%) | ATPase activity (%) |
|---|---|---|---|
| $MgCl_2$ medium | | | |
| Control $F_1$ | 100 | 100 | 100 |
| Photolabeled $F_1$ (1 μM $NAP_4$-Ado*PP*[NH]*P*) | 88 | 90 | 95 |
| (6 μM $NAP_4$-Ado*PP*[NH]*P*) | 32 | 71 | 70 |
| (46 μM $NAP_4$-Ado*PP*[NH]*P*) | 3 | 30 | 20 |
| EDTA medium | | | |
| Control $F_1$ | 100 | 100 | 100 |
| Photolabeled $F_1$ (6 μM $NAP_4$-Ado*PP*[NH]*P*) | 70 | 85 | 75 |
| (28 μM $NAP_4$-Ado*PP*[NH]*P*) | 20 | 70 | 55 |
| (82 μM $NAP_4$-Ado*PP*[NH]*P*) | 2 | 50 | 40 |

to 80%, the fluorescence response of aurovertin was 23% lower than with native $F_1$. In contrast to the binding of aurovertin to $F_1$, which was indeed not severely affected by photolabeling, the response to ADP (enhancement of fluorescence) and to ATP (quenching of fluorescence) were markedly inhibited in photolabeled $F_1$. Table IV shows data on the relationship between the percentage of inhibition of the ADP-induced enhancement of fluorescence, the ATP-induced quenching of fluorescence and the ATPase activity, when $F_1$ was photolabeled with $NAP_4$-Ado*PP*[NH]*P* to varying degrees. There was a parallelism between the inhibition of the ATP-induced quenching of fluorescence and the inhibition of the ATPase activity. On the other hand, the ADP-induced enhancement of fluorescence was far more sensitive to photolabeling than the ATPase activity. These effects were observed in either an $MgCl_2$- or EDTA-supplemented medium. They suggest that the quenching step reflects a molecular event related to the functioning of the catalytic site.

## Discussion

$NAP_4$-Ado*PP*[NH]*P* is a new photoactivable nucleotide to be added to the list of azido derivatives of ADP or ATP, which have been used so far to probe nucleotide-binding sites in mitochondrial, bacterial and chloroplast ATPases [9,13,14,22,27–37]. The interest of $NAP_4$-Ado*PP*[NH]*P* resides in the fact that its nucleotide moiety, Ado*PP*[NH]*P*, is not hydrolyzable. Therefore, binding of $NAP_4$-Ado*PP*[NH]*P* to $F_1$ can be investigated in an $MgCl_2$ medium, which was not feasible for $NAP_4$-ATP, since the latter was readily hydrolyzed by $F_1$ under these conditions [14]. All the photolabeling experiments described in this paper were performed with mitochondrial native $F_1$ which had not been depleted of its tightly bound nucleotides. It is therefore likely that $NAP_4$-Ado*PP*[NH]*P* binds predominantly to the exchangeable nucleotide-binding sites of the enzyme. Data were provided showing that $NAP_4$-Ado*PP*[NH]*P* retains the binding properties (affinity and specificity) of its parent nucleotide.

In many respects, the binding properties of $NAP_4$-Ado*PP*[NH]*P* are similar to those of $NAP_4$-ADP in either an $MgCl_2$- or EDTA-supplemented medium, and $NAP_4$-ATP in an EDTA medium [14,22]. For example, as with $NAP_4$-ADP, full photoinactivation of $F_1$ by $NAP_4$-Ado*PP*[NH]*P* was attained when about 2 mol photolabel had bound to 1 mol $F_1$. A similar stoichiometry was reported for photoinactivation of mitochondrial $F_1$ by 8-azido ATP [29]. Further, like for the binding of $NAP_4$-ADP to mitochondrial $F_1$ (unpublished data) or bacterial (*Escherichia coli*) $F_1$ [22], $NAP_4$-Ado*PP*[NH]*P* at low concentrations was found to bind preferentially to the $\alpha$-subunit rather than to the $\beta$-subunit, whether or not the medium was supplemented with $MgCl_2$. These data are consistent with the view that Ado*PP*[NH]*P* binds to the same site as ADP or ATP, and they clearly show that Ado*PP*[NH]*P* is an interesting, nonhydrolyzable substitute for ATP. At this point, we would like to discuss more thoroughly the significance of the covalent photolabeling of the $\alpha$- and $\beta$-subunits in terms of exploration and assignment of binding sites to specific subunits, and to call attention to ambiguities in interpreting photolabeling data with a potential multisite enzyme like $F_1$. The dual, but concentration-dependent labeling of $\alpha$- and $\beta$-subunits by $NAP_4$-Ado*PP*[NH]*P* (this paper) and also by $NAP_4$-ADP [22] cannot be interpreted unequivocally; different explanations are presented hereafter.

(1) The simplest explanation is that photolabeling of the $\alpha$- and $\beta$-subunits in $F_1$ by azido nucleotides corresponds to the presence of specific nucleotide-binding sites in both the $\alpha$- and $\beta$-subunits. The fact that the $\alpha$-binding sites are saturated prior to the $\beta$-binding sites would mean that the former bind nucleotides with higher affinity than the latter. Since the $\beta$-subunit contains the catalytic site [38], one may speculate that the high-affinity site present on the $\alpha$-subunits has a regulatory function. To explain the linear relationship between photolabeling of $F_1$ and the subsequent photoinactivation, together with the fact that the $\alpha$-subunits are photolabeled prior to the $\beta$-subunits, one has to admit that photolabeling of the $\alpha$-subunits and of the $\beta$-subunits is equivalent in its inactivating effect on $F_1$. This is in accordance with the fact that although the $\alpha$-subunit does not contain the catalytic site, its genetic alteration [39], or chemical modification [27,40] results in loss of the ATPase activity of $F_1$. Taking into account the preceding data, and considering that photolabeling of two sites per $F_1$ is required for full inactivation, it may be inferred that a molecule of $F_1$ can be inactivated, following photolabeling of either two $\alpha$-subunits, or two $\beta$-subunits or one $\alpha$- and one $\beta$-subunit.

(2) A closely related interpretation is that both the $\alpha$- and $\beta$-subunits possess nucleotide-binding sites, but the population of $F_1$ molecules is characterized by a topographical heterogeneity illustrated by a differential accessibility of the $\alpha$- and $\beta$-subunits to $NAP_4$-nucleotides. A fraction of the $F_1$ molecules would be characterized by a high reactivity of their $\alpha$-subunits to low concentrations of photolabel, concomitant with a low reactivity of their $\beta$-subunits, possibly due to shielding of the nucleotide sites of the $\beta$-subunits by juxtaposed $\alpha$-subunits [41]. In contrast, in the remaining fraction of $F_1$ molecules, the nucleotide sites of the $\beta$-subunits are supposed to be exposed to the external medium and would therefore be accessible to added $NAP_4$-nucleotides; the unmasked $\beta$-subunits would bind the photolabels with moderate affinity, as compared to the $\alpha$-subunits in the first fraction. In spite of this topographical heterogeneity, both types of $F_1$ molecules would be fully inactivated by photolabeling of either two $\alpha$-, or two $\beta$-, or one $\alpha$- and one $\beta$-subunits, as postulated above. This hypothesis deserves further consideration.

(3) A third interpretation of photolabeling data is that all exchangeable binding sites in $F_1$ are located on one subunit, for example, at the catalytic site of the $\beta$-subunit, and that the photolabeling of the other subunit, namely, the $\alpha$-subunit, results from artifacts inherent to the photolabel used. In $NAP_4$-nucleotides, the azido group is linked by a long chain to the nucleotide moiety. Assuming that the nucleotide moiety binds to the $\beta$-subunit, and that the $\alpha$- and $\beta$-subunits in $F_1$ are juxtaposed [42], one may imagine that the azido group at the end of the chain oscillates between two proximal [43] $\alpha$- and $\beta$-subunits; consequently, the nitreno group generated upon photoiradiation would bind to either the $\beta$- or $\alpha$-subunits. This interpretation is not likely, however,

since the two short-arm photolabels, azido-benzoyl-ADP and -ATP, also bind to the α- and β-subunits and inactivate $F_1$ with the same dose-effect relationship as $NAP_4$-ADP, $NAP_4$-ATP and $NAP_4$-Ado*PP*[NH]*P* [22]. It does not fit either with photolabeling of both the α- and β-subunits of $F_1$ by 8-azido-ATP and 8-azido-ADP, two photolabels in which the azido group is directly attached to the adenine ring of the nucleotide [9].

(4) Finally, one may imagine that the nucleotide-binding sites are located on one specific subunit of $F_1$, for example, the β-subunit, at the interface between this subunit and a juxtaposed α-subunit. At low concentrations of added arylazido nucleotide, in spite of the attachment of the nucleotide moiety to the β-subunit, the azido group could be preferentially directed to the proximal α-subunit for steric reasons, and the nitreno group generated by photoirradiation would therefore bind to this subunit. At higher concentrations of added photolabel, the interaction between the α- and β-subunits would loosen, due to possible conformational changes induced by prior binding of the photolabel to the β-subunit. Under these conditions the photolabeling of the β-subunit would become more selective. An extra assumption required for hypothesis 4 would be that the α-subunit is devoid of specific nucleotide site(s). This is unlikely in view of the demonstration (Refs 6 and 9 and this paper) that 1 mol $F_1$ is able to bind more than 5 mol adenine nucleotides. Since the $\alpha\beta$ stoichiometry of mitochondrial $F_1$ is likely $\alpha_3$ $\beta_3$, this strongly suggests that each α- and β-subunit can bind one molecule of adeninenucleotide. At the present time, a choice between the explanations discussed above is hardly feasible.

The response of the fluorescence intensity of the aurovertin-photolabeled $F_1$ complex to ADP and ATP deserves some comments. There was a clear parallelism between ATPase inactivation and the inhibiton of ATP-induced fluorescence quenching in $F_1$ samples photolabeled to different extents; this suggests that fluorescence quenching is related to the binding of ATP to the catalytic site. In contrast, the fluorescence enhancement induced by ADP was much more sensitive to photolabeling by $NAP_4$-Ado*PP*[NH]*P* than fluorescence quenching; fluorescence enhancement may therefore reflect binding of ADP to sites other than the catalytic ones; regulatory sites are likely candidates. This interpretation would be consistent with observations showing that just the opposite fluorescent response of the $F_1$-aurovertin complex to addition of ADP and ATP was obtained, namely, no effect on the ADP-induced stimulation and inhibiton of the ATP-induced quenching, when $F_1$ was modified by nitrobenzofurazan [44] and dicyclohexylcarbodiimide [45], two reagents which most likely interact at or close to the catalytic site of $F_1$.

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