Exploration of Adenosine 5′-Diphosphate–Adenosine 5′-Triphosphate Binding Sites of *Escherichia coli* Adenosine 5′-Triphosphatase with Arylazido Adenine Nucleotides†

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ABSTRACT: Interaction of [^{3}H]-3′-*O*-(4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl)adenosine 5′-diphosphate ([^{3}H]-NAP_4-ADP) and [^{3}H]-3′-*O*-(4-[*N*-(4-azido-2-nitrophenyl)-amino]butyryl)adenosine 5′-triphosphate [(^{3}H]NAP_4-ATP) with purified coupling factor 1 solubilized from *Escherichia coli* (BF_1) and isolated BF_1 subunits is described. The two arylazido nucleotides met the criteria of photoaffinity labels. In the dark, the ATP analogue was hydrolyzed by BF_1, and the ADP analogue competed with the BF_1 ATPase reaction. The nitrene generated by photoirradiation bound covalently to BF_1, and the binding was correlated with inactivation of the enzyme. Preincubation of BF_1 with ADP or ATP prevented the covalent photolabeling by NAP_4-ADP or NAP_4-ATP and the concomitant inactivation. Finally, the fluorescence response of the aurovertin–BF_1 complex to ADP and chiefly ATP was markedly altered in NAP_4-ADP-inactivated BF_1. All these data indicated that NAP_4-ADP and NAP_4-ATP are specifically recognized by the nucleotide site(s) of BF_1. In the dark, the stoichiometry of reversible binding was dependent on the ionic strength of the medium and the presence of divalent cations including Mg^{2+} and Mn^{2+}; in an ethylenediaminetetracetic acid (EDTA) supplemented medium or at low concentrations of $MgCl_2$ (<0.2 mM), one strong binding site (K_d = 3 μM) for NAP_4-ADP or NAP_4-ATP was revealed; at high concentrations of $MgCl_2$ (>5 mM), loose binding sites (K_d = 300 μM) were unmasked; the maximal number of unmasked loose sites per BF_1 was 2–3 mol/mol at 40–50 mM $MgCl_2$. 4-Chloro-7-nitrobenzofurazan, a reagent which interacts at or close to the catalytic site of *E. coli* BF_1 on the β subunit [Lunardi, J., Satre, M., Bof, M., & Vignais, P. V. (1979) *Biochemistry 18*, 5310–5316], interfered essentially with the NAP_4-ADP low-affinity binding. Upon photoirradiation with NAP_4-ADP or NAP_4-ATP in the presence of EDTA, full inactivation was achieved with 2 mol of bound photolabel/mol of BF_1. The distribution of the covalently bound photolabel between the α and β subunits depended on its initial concentration. When BF_1 was photoirradiated with high concentrations of NAP_4-ATP or NAP_4-ADP (75 μM) to yield nearly total inactivation, the α and β subunits were equally photolabeled; at low concentration of either photolabel (5 μM) for which inactivation was only 25–30%, the α subunit was preferentially photolabeled, indicating a higher affinity of the α subunit for ADP and ATP. Preincubation of BF_1 with ADP or ATP prior to photoirradiation with NAP_4-ADP or NAP_4-ATP interfered more with the photolabeling of the β subunit than with the photolabeling of the α subunit; preincubation with nitrobenzofurazan chiefly interfered with the photolabeling of the β subunit. The equal distribution of bound photolabel between the α and β subunits of purified BF_1, found under conditions of nearly full inactivation after irradiation of BF_1 with NAP_4-ADP and NAP_4-ATP in the presence of EDTA, was also observed after irradiation of BF_1 with NAP_4-ADP in the presence of $MgCl_2$. Similar photolabeling data were obtained with membrane-bound BF_1 in *E. coli* membrane particles. Photolabeling BF_1 with 3′-*O*-[4-azidobenzoyl]-ATP or 3′-*O*-[4-azidobenzoyl]-ADP, two short-arm photolabels, yielded data similar to those obtained with NAP_4-ADP and NAP_4-ATP. Photoirradiation of isolated α and β subunits from BF_1 with NAP_4-ADP and NAP_4-ATP resulted in the saturable covalent binding of ~1 mol of photolabel/mol of subunit α or β. The data suggest that the β subunit contains a loose site which could be the catalytic site of BF_1, whereas the α subunit contains a tight binding site, probably involved in regulation.

The adenine nucleotides bound to H^+-linked ATPases can be divided into two classes depending on their ability to exchange readily with externally added nucleotides. The nonexchangeable nucleotides may have a regulatory or structural function; the lack of exchangeability implies that they are bound to very tight sites. The exchangeable nucleotides may be involved in catalytic activity or in regulation. They are presumably located at moderately tight sites or loose sites. The photoactivable derivatives of ADP or ATP which have been recently used [for review cf. Guillory (1979)] to investigate the nucleotide sites of H^+-linked ATPases are believed to exchange with nucleotides bound to moderately tight or loose sites. These derivatives include 8-azido-ADP and 8-azido-ATP where the azido group is attached to the adenine ring of the nucleotide and the arylazido derivatives of ADP and ATP where the arylazido group is attached to the ribose moiety of ADP and ATP via a carbon chain.

A number of photolabeling experiments with the isolated coupling factors of mitochondrial ATPase (F_1)[1] (Russell et al., 1976; Lunardi et al., 1977; Wagenvoord et al., 1977, 1979; Cosson & Guillory, 1979) and chloroplastic ATPase (CF_1) (Schäfer et al., 1978; Carlier et al., 1979) have led to the

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[1] Abbreviations used: NAP_4-ADP, 3′-*O*-(4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl)adenosine 5′-diphosphate; NAP_4-ATP, 3′-*O*-(4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl)adenosine 5′-triphosphate; AP-ADP, 3′-*O*-(4-azidobenzoyl)adenosine 5′-diphosphate; AP-ATP, 3′-*O*-(4-azidobenzoyl)adenosine 5′-triphosphate; BF_1, soluble bacterial ATPase; F_1, soluble mitochondrial ATPase; CF_1, soluble chloroplastic ATPase; Nbf, 4-chloro-7-nitrobenzofurazan; DCCD, dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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conclusion that photoactivable ADP derivatives bind to both the α and β subunits of the coupling factors whereas photoactivable ATP derivatives may bind, under the appropriate conditions, preferentially to the β subunit. In contrast to F_1 and CF_1, the existing data on photolabeling of bacterial ATPase (BF_1) are both fragmentary and contradictory; they essentially concern 8-azido-ATP that was reported to bind preferentially to the α subunit of *Escherichia coli* BF_1 (Verheijen et al., 1978) and to the β subunit of *Micrococcus luteus* BF_1 (Scheurich et al., 1978). Since the position of the label on the nucleotide may be critical for recognition by ATPase, it was desirable to undertake complementary studies with the arylazido derivatives of ADP and ATP. The choice of these derivatives was in fact dictated by evidence that they are better affinity labels for F_1 and CF_1 than 8-azido-ADP and 8-azido-ATP; for example arylazido-ATP is hydrolyzed by F_1 30 times faster than 8-azido-ATP (Guillory, 1979); in the case of CF_1, arylazido-ADP competes with ADP with a much higher affinity (K_i = 6 μM) than 8-azido-ADP does (K_i = 190 μM) (Schäfer et al., 1978). Another consideration which led us to investigate in more detail the nucleotide sites in BF_1 is that this enzyme readily dissociates into subunits and the activity can be reconstituted by reassociation of the subunits. This opened the possibility to study with a hybrid enzyme reconstituted from photolabeled and unmodified subunits the function of nucleotide sites in each subunit. In the present paper, we report detailed photolabeling studies on *E. coli* BF_1 and its isolated subunits. The long-chain arylazido nucleotides, NAP_4-ADP and NAP_4-ATP, and the "zero-length" chain derivatives AP-ADP and AP-ATP were used. Special emphasis was directed toward the effect of cation concentration on the reversible binding of arylazido nucleotides to BF_1 in the dark and on factors which control the covalent photolabeling of the two major subunits, α and β, of BF_1.

Materials and Methods

Materials. Two types of arylazido nucleotides were used in this study. In the first one, the arylazido group was linked by a γ-aminobutyric chain to the ribose moiety of the nucleotides (NAP_4 derivatives), and in the second one, the arylazido group was directly attached to the ribose of ADP or ATP (AP derivatives). [^{3}H]NAP_4-ADP and [^{3}H]NAP_4-ATP were synthesized by the method of Jeng & Guillory (1975), slightly modified by Lunardi et al. (1977). [2,3-^{3}H]-4-Aminobutyric acid (45 mCi/μmol) was obtained from the CEA (Saclay, France). The final specific radioactivity of these analogues was 28 × 10^7 dpm/μmol. [^{14}C]AP-ADP and [^{14}C]AP-ATP (40 × 10^6 dpm/μmol) were synthesized by coupling *p*-[^{14}C]azidobenzoic acid and ADP or ATP as described for arylazido-ADP (Jeng & Guillory, 1975; Lunardi et al., 1977). *p*-[^{14}C]Azidobenzoic acid was prepared from *p*-[^{14}C]aminobenzoic acid (50 μCi/μmol) (CEA, Saclay, France), according to Hixson & Hixson (1975). [γ-^{32}P]ATP was obtained from the Radiochemical Center, Amersham, United Kingdom).

Aurovertin D was purified from cultures of *Calcarisporium arbuscula* (NRRL 3705) (Osselton et al., 1974) and stored at −20 °C as an ethanolic solution protected from light. The molar extinction coefficient of the aurovertin D preparation used in this work was 35 100 at 368 nm. Aurovertin fluorescence was measured at 30 °C with a Perkin-Elmer MPF 2A fluorimeter; the excitation wavelength was set at 365 nm and the emission wavelength at 470 nm.

Organism and Growth Conditions. The *E. coli* strain used in this study was strain AN 180 (*arg* E3, *thi*-1) (Butlin et al., 1971). Bacteria were grown at 37 °C on peptone–yeast extract medium (Miller, 1972), harvested, and stored at −80 °C.

BF_1 Purification. Membranes were prepared by disruption of the cells in a Sorvall-Ribi press (Roisin & Kepes, 1972). BF_1 was released from the membranes by chloroform treatment (Beechey et al., 1975). It was purified by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) at 4 °C using a 50–750 mM Tris-HCl gradient containing 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP, pH 7.4, and 20% methanol (v/v) (Vogel & Steinhart, 1976). Active fractions, eluted at ~0.2 M Tris-HCl, were pooled and concentrated by precipitation of the enzyme by poly(ethylene glycol) 6000. BF_1 was further purified by gel filtration on a Sepharose 6B column equilibrated with a buffer made of 50 mM Tris-HCl, 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP, pH 7.4, and 20% methanol (v/v). Purified BF_1 was stored at 0–4 °C at a protein concentration of ~10 mg/mL. The specific activity of purified BF_1 ranged between 25 and 35 μmol of ATP hydrolyzed min^{-1} mg^{-1} of protein at 30 °C. A molecular weight of 320 000 (Satre & Zaccaï, 1979) was assumed for calculation of binding data. Prior to binding assays, BF_1 samples were filtered through short Sephadex columns equilibrated with 50 mM Tris–acetate, pH 7.5, and $MgCl_2$ at the indicated concentration. The volume of BF_1 solution used in binding assays was between 5% and 10% of the final volume of the incubation medium.

Purification of BF_1 Subunits. Purified BF_1 was dissociated into subunits as described by Vogel & Steinhart (1976), except that freezing and thawing was repeated 3 times. The dissociated BF_1 in 50 mM Mops, 5 mM ATP, 1 M LiCl, and 2.5 mM 2-mercaptoethanol, pH 6.5 (buffer I), was dialyzed at 0 °C against 100 volumes of buffer II made of 50 mM Mops, 5 mM ATP, and 2.5 mM 2-mercaptoethanol, pH 6.5, for 2 h with three changes. The dialyzed sample of BF_1 was applied to a DEAE-cellulose column (DE-52 Whatman) equilibrated with buffer II. The column was washed with a small amount of buffer II and subsequently eluted with a 200-mL linear gradient of buffers I and II. The eluates were collected and protein fractions concentrated in an Amicon B15 cell. Essentially two fractions were obtained, one containing mainly the α subunit with small amounts of γ and ϵ subunits and the other containing the pure β subunit. There was no δ subunit, probably due to the chloroform extraction of BF_1 (Cox et al., 1978).

Assay of ATPase Activity. ATPase activity of BF_1 was routinely assayed at 30 °C with an ATP regenerating system. ATP hydrolysis was started by addition of an aliquot fraction of BF_1 (3–5 μg) in a medium containing 4 mM phosphoenolpyruvate, 20 μg of 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. After a 5-min incubation, the reaction was terminated by addition of 0.2 mL of trichloracetic acid (50% w/v). The phosphate released by ATP hydrolysis was estimated by the method of Fiske & SubbaRow (1925). Protein concentration was measured as reported by Bradford (1976) with bovine serum albumin as standard. In the specific experiment where the K_M for NAP_4-ATP and the K_i for NAP_4-ADP were determined, ATPase activity was assayed by an isotopic method using [γ-^{32}P]ATP as substrate. ^{32}P was extracted by the method of Nielsen & Lehninger (1955), and the radioactivity was measured in the extracts.

Reversible Binding of [^{14}C]ADP and [^{3}H]NAP_4-ADP to BF_1 in the Dark. Samples of BF_1 were incubated in the dark as detailed in Figures 1 and 2. After 30 min of incubation at 25 °C, the samples were filtered through small Sephadex G-50 (fine) columns following the elution–centrifugation

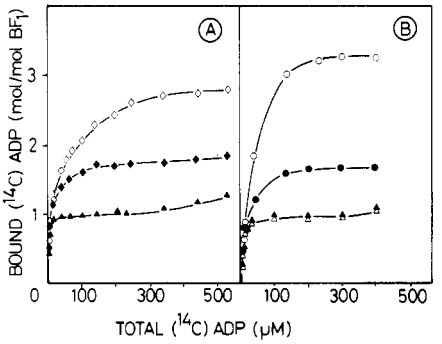

FIGURE 1: Titration of BF_1 with [^{14}C]ADP. (A) Effect of $MgCl_2$. BF_1 (40 μg) was incubated in 0.1 mL of 50 mM Tris–acetate, pH 7.5, supplemented with 2 mM EDTA (▲), 5 mM $MgCl_2$ (♦), or 40 mM $MgCl_2$ (◊), and [^{14}C]ADP at the indicated concentrations. (B) Effect of NaCl. BF_1 (40 μg) was incubated in 0.1 mL of 50 mM Tris–acetate, pH 7.5, supplemented with 2 mM EDTA (▲), 2 mM EDTA plus 115 mM NaCl (△), 10 mM $MgCl_2$ (●), or 10 mM $MgCl_2$ plus 115 mM NaCl (○) in the presence of [^{14}C]ADP at the indicated concentrations. All incubations were for 30 min at 25 °C. Separation of bound and free [^{14}C]ADP was performed as described under Materials and Methods.

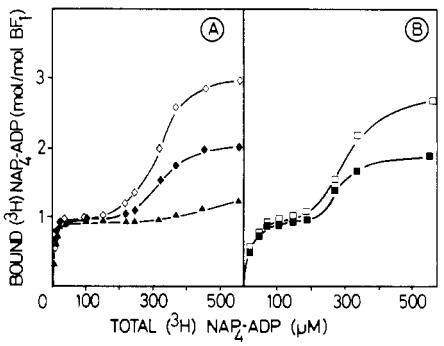

FIGURE 2: Titration of BF_1-ATPase in the dark by [^{3}H]NAP_4-ADP. (A) Effect of $MgCl_2$. BF_1 (40 μg) was incubated in 0.1 mL of a 50 mM Tris–acetate, pH 7.5, supplemented with 2 mM EDTA (▲), 5 mM $MgCl_2$ (♦), or 40 mM $MgCl_2$ (◊), and [^{3}H]NAP_4-ADP at the indicated concentrations. (B) Effect of Nbf. Control BF_1 (□) and Nbf-modified BF_1 (■) were incubated in 0.1 mL of a 50 mM Tris–acetate, pH 7.5, and 5 mM $MgCl_2$ buffer in the presence of NAP_4-ADP at the indicated concentrations. Nbf modification of BF_1 was obtained by incubation of BF_1 in the same medium for 30 min in the presence of 100 μM Nbf at 20 °C. All titrations with [^{3}H]-NAP_4-ADP were carried out at 25 °C in the dark to avoid covalent photolabeling. Blanks were carried out by adding an excess of ADP (5 mM) prior to photoirradiation to determine the nonspecifically bound [^{3}H]NAP_4-ADP.

method described by Penefsky (1977) to remove the free [^{14}C]ADP and [^{3}H]NAP_4-ADP; the bound radioactivity and the protein concentration of the filtrates were determined.

Photolabeling Assays. BF_1 was preincubated in the dark for 15 min at 25 °C in a medium made of 200 mM sucrose, 30 mM Tris–acetate, and 2 mM EDTA, pH 7.5, in the presence of [^{3}H]NAP_4-ADP or [^{3}H]NAP_4-ATP in small glass tubes. The tubes exposed to light irradiation (Osram lamp 250 W) were rotated horizontally at 150 rpm for 20–25 min in a thermostated bath at 23–25 °C [see Lunardi et al. (1977)]. Following photoirradiation, the samples were supplemented with 5 mM ADP or 5 mM ATP to displace the noncovalently bound arylazido nucleotides and then passed through short Sephadex G-50 (fine) columns, according to the elution–centrifugation method described by Penefsky (1977), to remove the photoirradiation products. To estimate the nonspecifically bound radioactivity, we performed blanks by adding a large excess of ADP or ATP (5 mM) to BF_1 samples prior to photoirradiation. When photolabeling was carried out with isolated BF_1 subunits, the noncovalently bound [^{3}H]-NAP_4-ADP or [^{3}H]NAP_4-ATP was removed by precipitating and washing the protein twice with cold trichloracetic acid (10% w/v final concentration).

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by Weber & Osborn (1969) using 10% acrylamide gels. Electrophoresis was carried out for 16 h by using 5 mA/gel. After staining and destaining, 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.

Results

Reversible Titration of ADP/ATP Sites of BF_1 by [^{3}H]-NAP_4-ADP and [^{14}C]ADP. Effect of Cations (Dark Reactions). In the dark, both [^{14}C]ADP and [^{3}H]NAP_4-ADP bound to BF_1. In both cases, the extent of binding was dependent on cation concentration. As shown in Figures 1 and 2, 1 mol of nucleotide binding sites/mol of BF_1 was titrated by [^{14}C]ADP or [^{3}H]NAP_4-ADP in the presence of EDTA at low ionic strength. Addition of $MgCl_2$ markedly increased the total amount of bound [^{14}C]ADP or [^{3}H]NAP_4-ADP. Maximal binding of both nucleotides, ranging between 3.2 and 3.6 mol/mol of BF_1, was obtained with 40–50 mM $MgCl_2$.

Table I: Binding Parameters of BF_1 with Respect to ADP and NAP_4-ADP (Reversible Binding in the Dark)[a]

		high-affinity sites		low-affinity sites	
ligand	$MgCl_2$ (mM)	n (mol/mol of BF_1)	K_d (μM)	n (mol/mol of BF_1)	K_d (μM)
[^{3}H]NAP_4-ADP	0	0.94	3.0		
[^{3}H]NAP_4-ADP	5	0.90	3.0	1.0	300
[^{3}H]NAP_4-ADP	40	0.84	2.6	2.0	300
[^{14}C]ADP	0	1.00	1.0		
[^{14}C]ADP	5	0.96	1.0	1.0	50
[^{14}C]ADP	40	0.98	3.2	2.0	50

[a] The binding data were obtained by treatment of Scatchard plots of Figures 1 and 2 according to Rosenthal (1967).

Raising the ionic strength by increasing the NaCl concentration did not alter [^{14}C]ADP binding in the presence of EDTA; it, however, enhanced the total amount of bound [^{14}C]ADP at a concentration of $MgCl_2$ (10 mM) which per se had a limited effect on [^{14}C]ADP binding (Figure 1B). The same observation held for [^{3}H]NAP_4-ADP binding. The enhancing effect of Mg^{2+} was shared by Mn^{2+}, but not by Ca^{2+}. The above results suggest that some divalent cations, including Mg^{2+} and Mn^{2+}, are able to unmask nucleotide binding sites and that this unmasking effect is amplified at high ionic strength.

The diphasic [^{3}H]NAP_4-ADP binding curve in the presence of $MgCl_2$ (Figure 2) indicated the presence of heterogeneous binding sites. This might be due to preexisting nonidentical sites or to identical sites whose binding parameters are controlled by negative cooperativity. In keeping with the alternative of preexisting nonidentical sites, two sets of sites (high affinity and low affinity) could fit the experimental data presented in Figures 1 and 2. The binding parameters of high- and low-affinity sites (Table I) were calculated after graphical analysis (Rosenthal, 1967) of the Scatchard plots of the binding data. The number of high-affinity sites (K_d = 1 μM for ADP and 3 μM for NAP_4-ADP) was limited to 1 mol/mol of BF_1; the number of low-affinity sites (K_d = 50 μM for ADP and 300 μM for NAP_4-ADP) amounted to 2–3 mol/mol of BF_1. The above titrations were restricted to ADP and NAP_4-ADP; the use of $MgCl_2$ precluded titrations with ATP

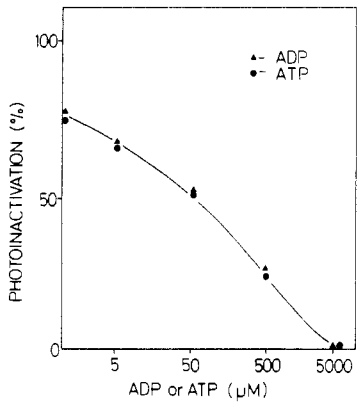

FIGURE 3: Photoinactivation of BF_1 by NAP_4-ADP and NAP_4-ATP. Protection by ADP and ATP. BF_1 (30 μg) was photoirradiated in 0.1 mL of a 200 mM sucrose, 20 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5, in the presence of 65 μM NAP_4-ADP or 65 μM NAP_4-ATP and ADP or ATP at the indicated concentrations.

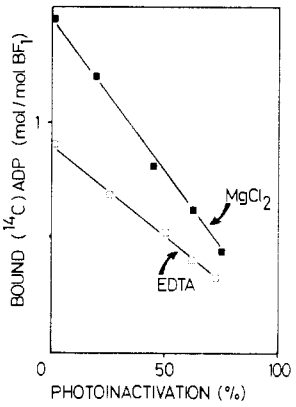

FIGURE 4: Effect of photoinactivation of BF_1 by NAP_4-ADP on its [^{14}C]ADP binding capacity. Samples of BF_1 were photoinactivated to different extents by successive additions of 10, 20, 40, and 60 μM [^{3}H]NAP_4-ADP, followed by photoirradiation. The photoirradiated BF_1 samples were incubated for 30 min at 25 °C in 0.1 mL of 50 mM Tris–acetate, pH 7.5, supplemented by 150 μM [^{14}C]ADP and either 2 mM EDTA or 2 mM $MgCl_2$. The bound [^{14}C]ADP was determinated after Sephadex filtration.

and NAP_4-ATP, because of the rapid hydrolysis of these nucleotides in the presence of $MgCl_2$.

Nbf, a reagent which is believed to bind at or near the catalytic site of ATPase (Ferguson et al.; 1975, Lunardi et al., 1979), did not modify the high-affinity binding of NAP_4-ADP but significantly decreased the low-affinity binding (Figure 2B), suggesting that the low-affinity binding sites may be involved in catalysis.

Photoinactivation and Covalent Photolabeling of BF_1 by [^{3}H]NAP_4-ADP and [^{3}H]NAP_4-ATP. Soluble BF_1 isolated from *E. coli* was readily inactivated when photoirradiated in the presence of NAP_4-ADP or NAP_4-ATP. That photoinactivation was due to interaction of the NAP_4-nucleotides with the ADP/ATP sites of BF_1 was shown by the following observations. (1) NAP_4-ATP was a substrate for BF_1; in the dark, it was hydrolyzed in the presence of $MgCl_2$ to give P_i; the rate of hydrolysis, 5–6 μmol of P_i min^{-1} mg^{-1}, was only 4–5 times less than that of ATP. The K_M for NAP_4-ATP was ~400 μM, a value close the K_M for ATP, 200–400 μM (Futai et al., 1974). (2) In the dark NAP_4-ADP inhibited competitively ATP hydrolysis by BF_1; the K_i for NAP_4-ADP was in the range of 140–200 μM; by comparison, the K_i for ADP was ~80 μM. (3) As previously shown (Table I), titration of BF_1 in the dark revealed one high-affinity binding site and two or three low-affinity sites for ADP and NAP_4-ADP. (4) Preincubation of BF_1 with ADP or ATP protected BF_1 from

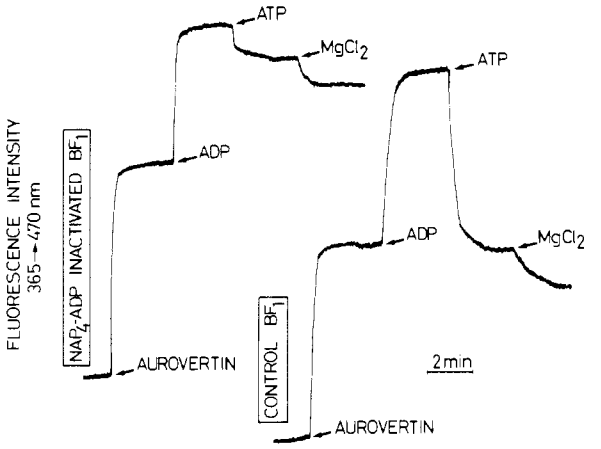

FIGURE 5: Effect of ADP, ATP, and $MgCl_2$ on the fluorescence intensity of aurovertin bound to native BF_1 and to BF_1 photolabeled by NAP_4-ADP. Assays were made at 30 °C; 150 μg of native BF_1 and photolabeled BF_1 (inactivated to 60%) were incubated with 1 μM aurovertin in 2 mL of a 200 mM sucrose, 20 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5; ADP, ATP, and $MgCl_2$ were added sequentially to final concentrations of 0.1, 3, and 2.5 mM, respectively.

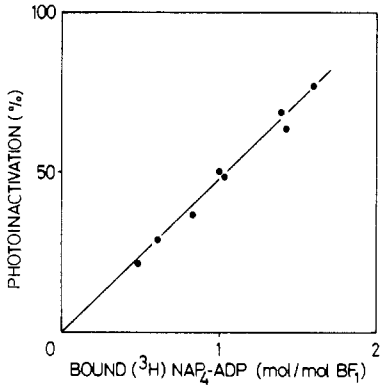

FIGURE 6: Correlation between photoinactivation and covalent photolabeling of BF_1 by [^{3}H]NAP_4-ADP. BF_1 (100 μg) in 0.1 mL of a 200 mM sucrose, 20 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5, was photoirradiated in the presence of varying concentrations of [^{3}H]NAP_4-ADP (10–400 μM). After Sephadex filtration, the bound radioactivity, the protein content, and the ATPase activity of the filtrates were determined.

photoinactivation by NAP_4-ADP (Figure 3). (5) Bound [^{14}C]ADP was decreased in BF_1 previously photolabeled with NAP_4-ADP; this was true for the binding of [^{14}C]ADP in either an EDTA- or $MgCl_2$-supplemented medium (Figure 4); (6) The last piece of evidence was based on the fluorescence response of aurovertin in photolabeled BF_1 (Figure 5). As shown by Satre et al. (1978, 1980), the fluorescence of aurovertin is enhanced when aurovertin binds to *E. coli* BF_1; the subunit which possesses the aurovertin site is the β subunit. Photolabeling of BF_1 with NAP_4-ADP did not prevent aurovertin binding; in fact, the enhancement of aurovertin fluorescence was virtually the same whether aurovertin was added to native BF_1 or to BF_1 photolabeled by NAP_4-ADP. The further enhancement of fluorescence caused by addition of ADP was somewhat inhibited in photoinactivated BF_1 as compared to the control, and the quenching effect obtained by further addition of ATP was strongly inhibited in photoinactivated BF_1 (Figure 5).

The extent of BF_1 photoinactivation varied with the concentration of NAP_4-ADP or NAP_4-ATP and the period of irradiation. As shown in Figure 6, there was a linear correlation between the percentage of inactivation and the amount of covalently bound [^{3}H]NAP_4-ADP, full inactivation corresponding to ~2 mol of [^{3}H]NAP_4-ADP bound/mol of en-

Table II: Effect of Concentration in [^{3}H]NAP_4-ATP and [^{3}H]NAP_4-ADP on the Distribution of Bound Radioactivity between the α and β Subunits of BF_1[a]

added ligand	total bound [^{3}H]NAP_4-ATP or [^{3}H]NAP_4-ADP (mol/mol of BF_1)	% inhibition of enzymatic act.	^{3}H radioactivity	
			α subunit (dpm)	β subunit (dpm)
[^{3}H]NAP_4-ATP (5 μM)	0.74	30	2140	350
[^{3}H]NAP_4-ATP (25 μM)	1.28	60	2880	1710
[^{3}H]NAP_4-ATP (75 μM)	1.60	85	3360	2420
[^{3}H]NAP_4-ADP (5 μM)	0.50	25	1400	390
[^{3}H]NAP_4-ADP (25 μM)	1.05	55	2540	1510
[^{3}H]NAP_4-ADP (75 μM)	1.40	68	2860	2500

[a] Photolabeling of BF_1 (40 μg) with [^{3}H]NAP_4-ATP or [^{3}H]NAP_4-ADP in 0.1 mL of 50 mM Tris-HCl and 2 mM EDTA, pH 7.5, was performed as follows. A first cycle of photoirradiation was performed for 20 min in the presence of 5 μM [^{3}H]NAP_4-ATP; this was followed by a new addition of [^{3}H]NAP_4-ATP to reach the final concentration of 25 μM [^{3}H]NAP_4-ATP and another photoirradiation cycle of 20 min; a final addition of [^{3}H]NAP_4-ATP was made to bring the final concentration of [^{3}H]NAP_4-ATP to 75 μM, accompanied by a 20-min photoirradiation. The same sequential procedure was adopted for photolabeling BF_1 with [^{3}H]NAP_4-ADP. The photolabeled BF_1 samples were processed as described under Materials and Methods. It was checked in control experiments that the enzymatic activity of BF_1 was virtually not modified (<5%) after three cycles of photoirradiation of 20 min each in the absence of photolabels.

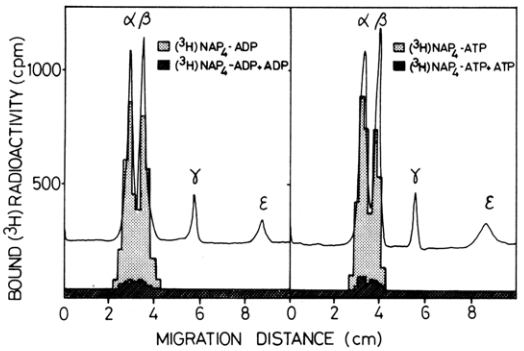

FIGURE 7: NaDodSO$_4$–polyacrylamide gel electrophoresis of BF_1 photolabeled with [^{3}H]NAP_4-ADP or [^{3}H]NAP_4-ATP. BF_1 (50 μg) was photoirradiated in 0.1 mL of a 200 mM sucrose, 30 mM Tris–acetate, and 2 mM EDTA, pH 7.5, buffer in the presence of 113 μM [^{3}H]NAP_4-ADP or 144 μM [^{3}H]NAP_4-ATP leading to 58 and 65% inactivation of ATPase, respectively. Blanks were carried out by adding 5 mM ADP or 5 mM ATP before photoirradiation. After Sephadex filtration, the BF_1 photoirradiated samples (10 μg) were subjected to NaDodSO$_4$ gel electrophoresis. The figure shows the densitometric tracing of the stained gels and the corresponding radioactivity pattern. The shaded area corresponds to the bound radioactivity still present after preincubation with 5 mM ADP or 5 mM ATP (blank).

zyme. A similar stoichiometry was observed for NAP_4-ATP (not shown). When used at concentrations higher than those yielding 100% inactivation, the amount of covalently bound [^{3}H]NAP_4-ADP or [^{3}H]NAP_4-ATP was in excess of 2 mol/mol of BF_1.

Distribution of Bound [^{3}H]NAP_4-ADP and [^{3}H]NAP_4-ATP in BF_1 Subunits. In all conditions tested, NaDodSO$_4$–polyacrylamide gel electrophoresis of the photolabeled BF_1 indicated that the covalently bound radioactivity was essentially located in the α and β subunits. However, the distribution of the bound radioactivity between the α and β subunits was found to depend on conditions of incubation. When BF_1 was photoirradiated with [^{3}H]NAP_4-ADP or [^{3}H]NAP_4-ATP in the presence of EDTA to yield 60–70% inactivation, the bound radioactivity was nearly equally distributed between the two subunits (Figure 7). Incubation with ADP or ATP prior to photoirradiation resulted in a marked decrease of bound radioactivity in both α and β subunits indicating that specific nucleotide sites had been photolabeled by [^{3}H]NAP_4-ATP or [^{3}H]NAP_4-ATP. Similar data were obtained when EDTA was replaced by 5 mM $MgCl_2$ and [^{3}H]NAP_4-ADP was used as photolabel. We did not attempt to photolabel BF_1 with [^{3}H]NAP_4-ATP in the presence of $MgCl_2$, since under these conditions NAP_4-ATP is hydrolyzed to give NAP_4-ADP.

Table III: Effect of Preincubation with ATP, ADP, or Nbf on the Respective Photolabeling of the α and β Subunits of BF_1[a]

added ligands	total bound [^{3}H]NAP_4-ATP or [^{3}H]NAP_4-ADP (mol/mol of BF_1)	^{3}H radioactivity	
		α subunit (dpm)	β subunit (dpm)
[^{3}H]NAP_4-ATP	0.84	2010	780
ATP + [^{3}H]NAP_4-ATP	0.70	1810	400
ADP + [^{3}H]NAP_4-ATP	0.67	1750	420
Nbf + [^{3}H]NAP_4-ATP	0.73	1960	360
[^{3}H]NAP_4-ADP	1.12	2700	1560
ATP + [^{3}H]NAP_4-ADP	0.95	2260	900
ADP + [^{3}H]NAP_4-ADP	0.75	1900	460
Nbf + [^{3}H]NAP_4-ADP	0.90	2380	580

[a] BF_1 (40 μg) was preincubated in the dark for 10 min with 20 μM ATP, 20 μM ADP, or 100 μM Nbf before photolabeling, with 14 μM [^{3}H]NAP_4-ATP or 20 μM [^{3}H]NAP_4-ADP.

The above covalent photolabeling data brought information on the localization of the nucleotide binding sites on BF_1 but not on their affinity. On the other hand, the dark binding reactions described in a preceding section indicated the presence of both strong and loose sites on BF_1 but did not provide information on their localization. The following photolabeling assays were set out to investigate the affinity of the α and β binding sites for NAP_4-ATP and NAP_4-ADP. As shown in Table II, varying the concentration of [^{3}H]-NAP_4-ATP and [^{3}H]NAP_4-ADP in the photoirradiation medium markedly altered the distribution of the covalently bound radioactivity between the α and β subunits. At a relatively high concentration of [^{3}H]NAP_4-ATP and [^{3}H]-NAP_4-ADP, 75 μM, resulting in large inactivation of BF_1 (85% and 68%, respectively), the amounts of bound photolabels were 1.60 and 1.40 mol/mol of BF_1, and the bound radioactivity was about equally distributed between the α and β subunits, with a slight predominance on the α subunit. As the concentration of the photolabels was decreased, the bound radioactivity tended to be concentrated in the α subunit; for example, at 5 μM [^{3}H]NAP_4-ATP and [^{3}H]NAP_4-ADP, the amounts of bound photolabels were 0.74 and 0.50 mol/mol of BF_1, and the bound photolabels were largely localized in the α subunit (86% and 78%, respectively). These data suggest that the α subunit in BF_1 binds NAP_4-ATP and NAP_4-ADP with a higher affinity than the β subunit. When photolabeling of BF_1 by [^{3}H]NAP_4-ATP or [^{3}H]NAP_4-ADP was conducted in the presence of a low concentration (20 μM) of ATP or ADP, the decrease in covalently bound radioactivity was more

marked in the β than in the α subunit (Table III). We also tested the effect of Nbf on photolabeling of BF_1 by [^{3}H]-NAP_4-ATP and [^{3}H]NAP_4-ADP (Table III). The incubation conditions were such that the enzyme was inactivated to only 70–75% by Nbf; under these conditions of limited inactivation, Nbf binds predominantly to the β subunit of BF_1 (Lunardi et al., 1979). As shown by data in Table III, Nbf prevented much more the photolabeling of the β subunit than that of the α subunit (54% vs. 2% in the case of [^{3}H]NAP_4-ATP, 63% vs. 12% in the case of [^{3}H]NAP_4-ADP).

Complementary photolabeling experiments were carried out with membrane-bound BF_1. Disrupted *E. coli* cells were photoirradiated in the presence of [^{3}H]NAP_4-ADP and EDTA to yield ~80% inactivation. After photoirradiation, BF_1 was isolated by the chloroform extraction procedure of Beechey et al. (1975) and analyzed for bound radioactivity. The amount of bound [^{3}H]NAP_4-ADP was 1.6 mol/mol of BF_1, and the radioactivity was equally distributed between the α and β subunits, a result very similar to that obtained with free BF_1 photoirradiated under similar conditions. At this point, it may be recalled that the δ subunit is lacking in free BF_1 and is present in membrane-bound BF_1. The similar photolabeling data obtained with free and bound BF_1 indicate that the δ subunit is not essential in photolabeling of the α and β subunits of BF_1.

Distribution of [^{14}*C*]*AP-ADP and* [^{14}*C*]*AP-ATP in* BF_1 *Subunits.* Because of the length of the γ-aminobutyric acid arm which links the ribose moiety of ADP or ATP to the azido group in NAP_4-ADP or NAP_4-ATP, it could be argued that the photolabeling data obtained with the above photolabels do not reflect the exact location of nucleotide sites. One may for example imagine that the nucleotide moiety of the NAP_4 derivatives interacts with only one subunit of BF_1; the distant azido group could bind to the same subunit, but it could bind to the adjacent subunit as well. To obviate this criticism, we synthesized two short-arm photolabels, AP-ADP and AP-ATP, in their ^{14}C-labeled form and used them at a concentration of 80 μM in the presence of EDTA to photolabel BF_1. The enzyme was photoinactivated to ~70%. The radioactivity profile after $NaDodSO_4$–polyacrylamide gel electrophoresis was similar to that obtained with [^{3}H]NAP_4-ADP and [^{3}H]NAP_4-ATP (Figure 7), which demonstrates that both the α and β subunits of BF_1 possess specific ADP/ATP sites capable of recognizing and binding photoactivable analogues.

Photolabeling of Isolated Subunits of BF_1. Two fractions were prepared from dissociated BF_1, one containing essentially the α subunit with small amounts of γ and ϵ subunits (fraction I) and the other one corresponding to the pure β subunit (Vogel & Steinhart, 1976). The two fractions were photoirradiated in the presence of [^{3}H]NAP_4-ADP and then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Both fractions bound radioactivity. In fraction I, the radioactivity was essentially located in the α subunit. Photolabeling of the α or β subunit was largely prevented by incubation with ADP or ATP prior to photoirradiation in the presence of [^{3}H]-NAP_4-ADP (Figure 8), which points to the specific labeling of ADP/ATP sites in the isolated α and β subunits. Successive cycles of photoirradiation of the isolated subunits in the presence of [^{3}H]NAP_4-ADP resulted in an increased binding of radioactivity, which tended toward a plateau corresponding to the binding of ~1 mol of photolabel/mol of subunit (Table IV). It must be noted that the long photoirradiation cycles used in this experiment had no deleterious effect per se on the subunits; in fact, fraction I and the β subunit subjected to light irradiation in the absence of photolabels could recombine, after mixing, to yield an enzyme whose activity was similar to that of BF_1 reconstituted from nonirradiated subunits.

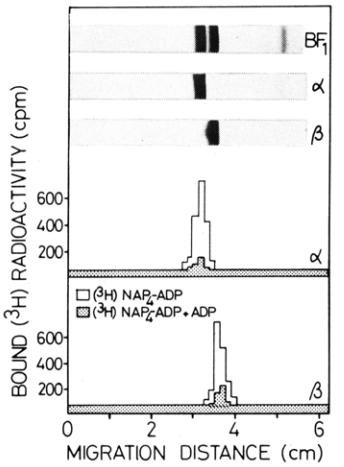

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of BF_1–isolated subunits photolabeled with [^{3}H]NAP_4-ADP. Two fractions were prepared from the dissociated BF_1. Fraction I was made of the α subunit and traces of γ and ϵ subunits; fraction II was the pure β subunit. Each fraction (30 μg) was photoirradiated for 20 min in 0.05 mL of a 200 mM sucrose, 30 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5, in the presence of 200 μM [^{3}H]NAP_4-ADP. After Sephadex filtration, aliquots of 8 μg of protein were subjected to $NaDodSO_4$ gel electrophoresis. The figure shows the gels after Coomassie blue staining (a native BF_1 sample is included) and the corresponding radioactivity profiles. The shaded area is the bound radioactivity remaining after preincubation with 5 mM ADP (blank). The specifically bound radioactivity, obtained by subtracting the blank from the total bound radioactivity, corresponded to 0.40 and 0.35 mol of [^{3}H]NAP_4-ADP/mol of α and β subunits, respectively.

Table IV: Binding Stoichiometry of [^{3}H]NAP_4-ADP to the Isolated BF_1 Subunits[a]

no. of photo-irradiation cycles	[^{3}H]NAP_4-ADP bound to fraction I (mol/mol)	[^{3}H]NAP_4-ADP bound to β subunit (mol/mol)
1	0.39	0.36
2	0.78	0.71
3	1.21	0.91
4	1.30	0.98

[a] Two fractions, one essentially made of the α subunit and traces of γ and ϵ subunits (fraction I) and the other made of the purified β subunit, were prepared from BF_1 by the procedure of Vogel & Steinhart (1976). 400 μg of each fraction was incubated separately in 0.6 mL of a 200 mM sucrose, 30 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5, in the presence of 40 μM [^{3}H]arylazido-ADP. Photoirradiation was carried out in four successive cycles of 20 min each. After each photoirradiation cycle, the samples were filtered through Sephadex G-50 columns. The protein content and the radioactivity were determined on the filtrates. Binding data were calculated by assuming a molecular weight of 56 000 for the α subunit and 52 000 for the β subunit (Vogel & Steinhart, 1976).

Discussion

Bragg & Hou (1977), using ammonium sulfate precipitation and Millipore filtration, investigated the binding parameters of ADP in *E. coli* BF_1; the binding stoichiometry was 0.63 mol of ADP/mol of enzyme, and the K_d value was 9.3 μM. Dunn & Futai (1980) have recently examined by equilibrium dialysis the ability of isolated subunits from *E. coli* BF_1 to bind ATP or ADP. They presented evidence that only the α subunit was able to bind ATP and ADP (~1 mol/mol of subunit α); the K_d values were 0.1 μM for ATP and 0.9 μM for ADP, which are typical to tight binding sites. Since *E. coli* BF_1 contains 3 mol of tightly bound nucleotide/mol of enzyme (Maeda et al., 1976), it was concluded that each α subunit in BF_1 con-

tained a single tight exchangeable nucleotide site, which is in agreement with an $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry (Kagawa et al., 1979). The tight binding site of the α subunit is probably not the catalytic site since the K_M for ATP hydrolysis is as high as 200–400 μM (Futai et al., 1974).

Our data on the reversible binding of ADP and its NAP_4 analogue (dark reaction) revealed that the amount of titrable nucleotide sites in *E. coli* BF_1 depended on the composition of the medium and that new saturable nucleotide sites could be unmasked at high ionic strength and, more specifically, at high Mg^{2+} and Mn^{2+} concentrations. In the presence of EDTA, only one tight site capable of binding ADP (K_d = 1 μM) or NAP_4-ADP (K_d = 3 μM) was titrated; addition of high concentrations of $MgCl_2$ or $MnCl_2$ elicited a maximum of two to three new saturable binding sites for ADP (K_d = 50 μM) or NAP_4-ADP (K_d = 300 μM). The mechanism of the unmasking effect of Mg^{2+} can only be speculated. It could be due to conformational changes in BF_1, resulting from neutralization by Mg^{2+} of negative charges of dicarboxylic amino acids residues. This is consistent with the reported interaction of Mg^{2+} with F_1 leading to a slow conformational transition of mitochondrial F_1 (Moyle & Mitchell, 1975; Hackney, 1979).

Upon photoinactivation, NAP_4-ADP and NAP_4-ATP bound covalently to BF_1, and concomitantly the ATPase activity of BF_1 was decreased. Full inactivation was accompanied by the binding of 2 mol of photolabel/mol of BF_1; the photolabel was present exclusively in the α and β subunits and equally distributed between the two subunits. The short-arm photolabels, AP-ADP and AP-ATP, also bound to the α and β subunits, which strengthens the conclusion that both the α and β subunits contain specific nucleotide sites capable of binding ADP or ATP. If we refer to our reversible titration data showing only 1 tight binding site/mol of BF_1 in the presence of EDTA, it is surprising that both the α and β subunits of BF_1, i.e., at least 2 sites/BF_1, are photolabeled by NAP_4-ADP or NAP_4-ATP also in the presence of EDTA. One must, however, keep in mind that covalent photolabeling is able to reveal not only tight but also loose sites. At this point, it is important to state that NAP_4-ADP and NAP_4-ATP bind covalently only to the sites which can exchange their bound nucleotides (tight or loose) against external nucleotides. In contrast, the tight nonexchangeable nucleotide sites are not expected to be readily photolabeled. That the α subunit contains a much tighter exchangeable binding site than the β subunit is based on at least three lines of evidence (1) Nbf, a ligand which binds to the β subunit of BF_1 (Lunardi et al., 1979), inhibited the reversible binding (dark reaction) of NAP_4-ADP to the loose site of BF_1 but not to the tight site (Figure 2); (2) the α subunit of BF_1 was preferentially photolabeled by low concentrations of NAP_4-ADP or NAP_4-ATP (Table III); (3) photolabeling of the β subunit of BF_1 by NAP_4-ADP or NAP_4-ATP was decreased more than that of the α subunit by preincubation of BF_1 with ADP or ATP. The loose binding site of the β subunit might be the catalytic site of BF_1. The localization of the catalytic site in the β subunit of BF_1 was demonstrated by inactivation of the enzyme upon covalent binding of DCCD and Nbf to the β subunit (Satre et al., 1979; Lunardi et al., 1979).

At the only concentration of 8-azido-ATP used in their experiments, 2 mM, Verheijen et al. (1978) found that the α subunit was photolabeled to 72% and the β subunit to 28%. Clearly this result resembles that obtained in our work with the lowest concentration of NAP_4-ATP or NAP_4-ADP (5 μM). When the concentration of NAP_4-ATP or NAP_4-ADP was raised, the α and β subunits became equally labeled. The difference between 8-azido-ATP and the arylazido analogues is probably due to the much higher affinity of the arylazido analogues for BF_1. The fact that F_1 and CF_1 exhibit a higher affinity for arylazido nucleotides than for 8-azido nucleotides was already reported by Guillory (1979) and Schäfer et al. (1978).

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Transient and Steady-State Kinetic Studies of Sodium–Potassium Adenosine Triphosphatase Using β-(2-Furyl)acryloyl Phosphate as Chromophoric Substrate Assay†

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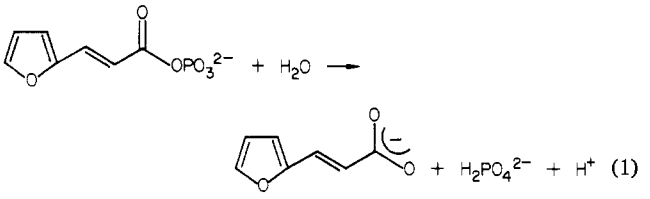
ABSTRACT: A convenient and highly specific continuous spectrophotometric assay for sodium–potassium adenosine triphosphatase activity utilizing the rapidly hydrolyzed and high-affinity chromophoric substrate β-(2-furyl)acryloyl phosphate (FAP) is described. The Na/K-ATPase-catalyzed hydrolysis of FAP is faster than that for ATP under all ionic conditions. The rate is neither inhibited nor activated by Na^+; it is dependent on $[K^+]$ and on $[Mg^{2+}]$. The hydrolysis of FAP to furylacrylate is accompanied by a large shift in the UV absorbance maximum. The spectrum of FAP, but not furylacrylate, is sensitive to noncovalent ligation with Mg^{2+}, a happenstance which permits the identification of Mg^{2+}FAP, and consequently allows for a probe of the role of Mg^{2+} in the catalysis. Mg^{2+} binding to the active site is essential for catalysis. MgFAP is more tightly bound to the site than is FAP^{2-}, but the complex is not obligatory for catalysis. The formation of a phosphoryl-enzyme intermediate is not evident in the reaction of FAP with the enzyme. Transient kinetic experiments, utilizing an excess of MgFAP, demonstrate a unique steady-state rate-limiting production of furylacrylate. These results indicate that the pathway demonstrated with ATP is not appropriate to the FAPase mechanism. The results suggest that acyl phosphates are good "phosphatase" substrates either because they are analogues of the phosphatase-specific phosphoryl-enzyme or because they react exclusively with the isomerized "E_2" form of the enzyme.

Ouabain-sensitive Na/K-ATPase[1] activity is conventionally measured by either quenched assays of the extent of inorganic phosphate formation or continuous assay via an ADP-dependent coupled enzymic process. The enzyme-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP) has been used as a direct and continuous spectrophotometric assay for the K^+- and Mg^{2+}-dependent activity of Na/K-ATPase (Glynn & Karlish, 1975). The catalyzed hydrolysis of pseudosubstrates such as PNPP (the "phosphatase" activity) does not require sodium. Not surprisingly, the rate of catalyzed hydrolysis of PNPP is much slower than that of ATP. Recent studies with crab nerve Na/K-ATPase have shown that two other synthetic organic phosphates, 2,4-dinitrophenyl phosphate (DNPP) and β-(2-furyl)acryloyl phosphate (FAP), are enzymically hydrolyzed at a faster rate than ATP (Gache et al., 1976, 1977). The hydrolysis of these latter pseudosubstrates is unaffected by sodium concentrations up to that optimal for ATPase activity. At sodium and potassium concentrations optimal for ATP hydrolysis, FAP is hydrolyzed 3–4 times faster than ATP and twice as fast as DNPP. The enzymic hydrolysis of these chromophoric organic phosphates, like ATP hydrolysis, is inhibited by the cardiac glycosides ouabain and strophanthidin (Gache et al., 1977). The large difference in absorption spectrum between the product (furylacrylate anion) and the substrate (eq 1) facilitates the



(1)

analysis of the extent of hydrolysis (Malhotra & Bernhard, 1968). The kinetics of enzyme-catalyzed hydrolysis of FAP ("FAPase") in the transient and the steady state has been investigated in order to determine suitability of FAP for mechanistic studies as well as for routine quantitative Na/K-ATPase assays.

The results we present define such a routine quantitative assay. These results show that two less obvious factors merit

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[1] Abbreviations used: Na/K-ATPase, sodium–potassium-activated adenosine triphosphatase, EC 3.6.1.3; Ca^{2+}-ATPase, calcium-activated adenosine triphosphatase, EC 3.6.1.3; FAP, β-(2-furyl)acryloyl phosphate; PNPP, *p*-nitrophenyl phosphate; DNPP, 2,4-dinitrophenyl phosphate; Cl_3CCO_2H, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenedinitrilotetraacetic acid; P_i, inorganic phosphate; DMF, dimethylformamide.

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