Exploration of nucleotide binding sites in the mitochondrial membrane by 2-azido-[α-^{32}P]ADP

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The ADP/ATP carrier of beef heart mitochondria is able to bind 2-azido-[α-^{32}P]ADP in the dark with a K_d value of $\simeq$ 8 μM. 2-Azido ADP is not transported and it inhibits ADP transport and ADP binding. Photoirradiation of beef heart mitochondria with 2-azido-[α-^{32}P]ADP results mainly in photolabeling of the ADP/ATP carrier protein; photolabeling is prevented by carboxyatractyloside, a specific inhibitor of ADP/ATP transport. Upon photoirradiation of inside-out submitochondrial particles with 2-azido-[α-^{32}P]ADP, both the ADP/ATP carrier and the β subunit of the membrane-bound F_1-ATPase are covalently labeled. The binding specificity of 2-azido-[α-^{32}P]ADP for the β subunit of F_1-ATPase is ascertained by prevention of photolabeling of isolated F_1 by preincubation with an excess of ADP.

Nucleotide binding *Mitochondrial membrane* *2-Azido-[α-^{32}P]ATP*

1. INTRODUCTION

A large number of studies have been devoted to the application of photoactivable derivatives of ADP or ATP to mitochondrial, bacterial and chloroplast ATPases. The two classes of azido derivatives most commonly used have an azido group placed either in the 8-position of the adenine ring or in the 3′-position of the ribose moiety. Substitution at the 8-position shifts the nucleotide conformation about the N-glycosidic linkage from *anti* to *syn*; this hinders the recognition of the nucleotide binding sites by 8-azido ADP or 8-azido ATP, since ADP and ATP have a non-fixed *anti* conformation. On the other hand, in the 3′-aryl-azido ADP or ATP derivatives, the azido group is attached to an aromatic ring which is linked to the 3′ carbon atom of the ribose most often by a butyryl or propionyl chain. This flexible chain is free to move within and around the nucleotide binding site, and the covalent link may therefore be located outside the nucleotide site.

The recently introduced 2-azido[β-^{32}P]ADP [1,2] obviates the difficulty encountered with the 3′-arylazido and 8-azido derivatives. In fact, the 2-azido nucleotides are expected to adopt preferentially an *anti* conformation [1], as do the natural nucleotides [3]. A minor disadvantage of 2-azido nucleotides is their spontaneous isomerization to form a tetrazole ring [4]. 2-Azido-[β-^{32}P]ADP has been applied to chloroplast F_1 and found to bind to a tight site on the β subunit [1]. This report concerns the photolabeling of two nucleotide binding proteins in the mitochondrial membrane by 2-azido-[α-^{32}P]ADP, namely the ADP/ATP carrier and the F_1 sector of the ATPase complex.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; CATR, carboxyatractyloside; BA, bongkrekic acid; PEI cellulose, polyethyleneimine cellulose

2. MATERIALS AND METHODS

2.1. *Biological preparations*

Beef heart mitochondria were prepared according to [5], and rat heart mitochondria as described

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in [6]. Inside-out sonic particles from beef heart mitochondria were prepared as described in [7]. Beef heart mitochondrial F_1-ATPase was purified and stored as an ammonium sulfate precipitate [8].

2.2. *Synthesis of 2-azido ADP*

2-Azido adenosine synthesized as in [9] was used to prepare the 2′,3′-*O*-isopropylidene-2-azido adenosine [10]. 2-Azido AMP was obtained from H_3PO_4 and 2′,3′-*O*-isopropylidene-2-azido adenosine as in [11]. Starting from 2-azido AMP imidazolidate and the tri-*n*-butylammonium salt of P_i, 2-azido ADP was prepared according to [12]. Its purity was checked by TLC on PEI cellulose, using 2 M Na formate (pH 3.6) as developing solvent; its R_f was 0.22. For the synthesis of 2-azido-[α-^{32}P]ADP, $H_3{}^{32}PO_4$ was used in the step leading to the preparation of 2-azido-[α-^{32}P]AMP.

2.3. *Biological assays*

The rate of [^{14}C]ADP transport in rat heart mitochondria was measured at 0°C by the direct exchange procedure [13], using a standard incubation medium consisting of 0.075 M sucrose, 0.225 M mannitol, 0.5 mM EDTA, 5 mM Tris, pH 7.3. The reaction was started by addition of [^{14}C]ADP and terminated after 15 s with 20 μM CATR. Unspecific binding of [^{14}C]ADP was assessed from the amount of [^{14}C]ADP incorporated in the presence of 20 μM CATR added prior to the azido derivative.

To determine the number and the affinity of binding sites for 2-azido-[α-^{32}P]ADP on the membrane-bound ADP/ATP carrier, rat heart mitochondria were incubated at 0°C in the dark with increasing concentrations of 2-azido-[α-^{32}P]ADP in the absence or presence of 20 μM CATR. Two parallel series of incubation were run in the presence of CATR: in the first one, CATR was added to the mitochondrial suspension, 10 min prior to 2-azido-[α-^{32}P]ADP and incubation was continued for 50 min. In the second series, CATR was added 30 min after addition of the azido derivative and incubation was continued for 30 min. The control assay with 2-azido-[α-^{32}P]ADP was for 60 min. Under all conditions equilibrium between free and bound ligands was fully achieved. Incubation was carried out with 0.25 mg mitochondrial protein and 0.5 ml standard medium in centrifuge tubes. The difference between the control curve and the inhibition curve with CATR added after the azido derivative corresponded to the specific binding of 2-azido-[α-^{32}P]ADP. Should transport have occurred, one would expect a difference between the two inhibition curves (CATR added after and prior to the azido derivative). After centrifugation, the pellets were digested in 1 ml of 5% Triton X-100 and 0.5 M NaCl, and radioactivity was assayed by liquid scintillation.

Displacement of the specifically bound [^{14}C]ADP by 2-azido ADP was determined as follows. Rat heart mitochondria (1 mg protein per ml standard medium) were preincubated with 20 μM [^{14}C]ADP for 1 h at 0°C. A first sample of the suspension was withdrawn and centrifuged to sediment the mitochondria. A second sample was withdrawn and left to incubate with CATR for 45 min at 0°C. Eight other samples were incubated for 45 min at 0°C with concentrations of 2-azido ADP ranging from 10 to 100 μM. Incubation was terminated by centrifugation. The difference of radioactivity in the pellets between samples 1 and 2 corresponded to the carried-bound ^{14}C (specifically bound [^{14}C]ADP). The difference between sample 1 and the samples of the series involving different concentrations of 2-azido ADP corresponded to bound [^{14}C]ADP removed by 2-azido ADP.

2.4. *Photolabeling assays and separation of the photolabeled peptides*

Photoirradiation was carried out with a XBO Xenon lamp 1000 W equipped with a parabolic reflector. Five ml of the mitochondrial or submitochondrial suspension in the standard medium at 1 mg/ml was preincubated for 15 min at 0°C in the dark with 40 μM 2-azido-[α-^{32}P]ADP and pipetted in a Petri dish placed on crushed-ice at a distance of 10 cm from the light source, under a flow of argon. After irradiation for 1 min, the particles were recovered by centrifugation. The pellet was rinsed with cold water, and the particles were lysed by heating for 2 min in a medium made of 0.1 M Tris–HCl (pH 7), SDS 2%, glycerol 10% and mercaptoethanol 2.5%. Ten μl aliquots of the lysate corresponding to about 50–100 μg protein were subjected to a SDS–polyacrylamide gel electrophoresis (PAGE) [14]. Electrophoresis was carried out for 4 h at 40 mA. Staining and destaining were conducted as described in [15]. For fluoro-

graphy, the gel plates were immersed in a bath of En^{3}Hance, then dried and applied to a Fuji RX film.

Beef heart mitochondrial F_1-ATPase (1 mg/ml) was incubated at room temperature with 40 μM 2-azido-[α-^{32}P]ADP in a buffer consisting of 0.27 mM sucrose, 0.05 M Tris-acetate and 1 mM $MgCl_2$, final pH 7.4, for 10 min in the dark, and then photoirradiated for 5 s as described for mitochondrial particles. Ten μg aliquots of photolabeled F_1 were subjected to SDS polyacrylamide slab gel electrophoresis [14], and urea slab gel electrophoresis [8]. Identification of F_1 subunits was based on the fact that the α, γ and ϵ subunits bind [^{14}C]NEM [16] and the β subunit binds [^{14}C]DCCD [17].

3. RESULTS

3.1. *Specific binding of 2-azido-[α-^{32}P]ADP to the ADP/ATP carrier*

In preliminary experiments (not shown), it was found that 2-azido-[α-^{32}P]ADP is not transported into rat heart mitochondria. In the absence of photoactivation, it binds to the ADP/ATP carrier in a reversible manner as illustrated by the following titration experiment carried out in the dark with increasing concentrations of 2-azido-[α-^{32}P]ADP (cf. section 2). The difference between the control titration curve and the curve corresponding to the addition of CATR after that of 2-azido-[α-^{32}P]ADP gave the specific binding for 2-azido-[α-^{32}P]ADP (fig.1). Similar results were obtained when CATR was added prior to 2-azido-[α-^{32}P]ADP. This corroborates that 2-azido-[α-^{32}P]ADP is not transported. A high affinity binding region could be recognized in the specific binding curve (inset) which corresponded to a number of sites of 0.8 nmol/mg protein and a K_d value of $\simeq 8\,\mu$M, a value very close to that found for the high affinity binding site of ADP [18]. The second region of the curve showed much less affinity and was apparently non-saturable; this apparent lack of saturation may be due to the tetrazole isomers in equilibrium with the azido derivative.

3.2. *Inhibitory effect of 2-azido ADP on ADP transport and ADP binding*

The present experiment, like the preceding one, was carried out in the dark to determine a possible

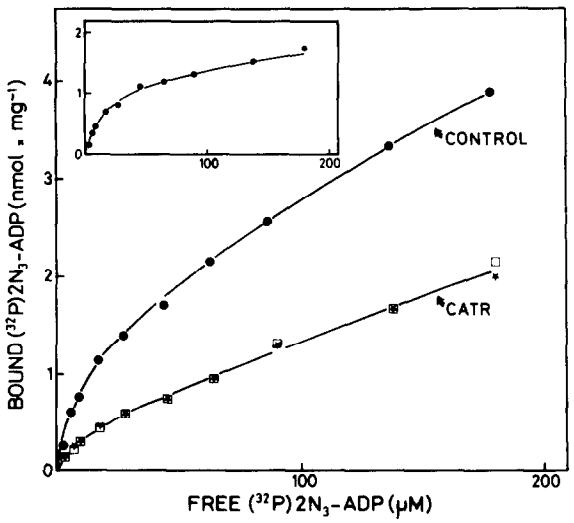

Fig.1. Binding of 2-azido-[α-^{32}P]ADP to the membrane-bound ADP/ATP carrier in rat heart mitochondria. Rat heart mitochondria (0.25 mg protein) were incubated in the dark in 0.5 ml of standard medium for 60 min at 0°C with increasing concentrations of 2-azido-[α-^{32}P]ADP. Removal of 2-azido-[α-^{32}P]ADP from the carrier sites was achieved by addition of 20 μM CATR (cf. section 2). The difference between the binding curves obtained in the absence and presence of CATR (specific binding sites) is shown in the inset. Note that similar binding data are observed when CATR is added prior to (★) or after (□) 2-azido-[α-^{32}P]ADP.

competitive inhibition of 2-azido ADP with respect to [^{14}C]ADP transport in heart mitochondria (fig.2). The reciprocal plot of the rate of [^{14}C]ADP transport vs the concentration of [^{14}C]ADP at different concentrations of 2-azido ADP indicated that 2-azido ADP behaved as a competitive inhibitor at high concentrations of [^{14}C]ADP. However, at low concentration of [^{14}C]ADP, the plot departed from linearity indicating complex interaction of 2-azido ADP with the vectorial reaction of [^{14}C]ADP transport and/or with the binding step. A possible effect of 2-azido ADP on [^{14}C]ADP binding was tested in another experiment (fig.3) where 2-azido ADP was added in the dark to mitochondria preincubated with [^{14}C]ADP. The total amount of carrier-bound [^{14}C]ADP was assessed by the release of [^{14}C]ADP upon addition of CATR. 2-Azido ADP removed bound [^{14}C]ADP in a dose-dependent, but not linear, fashion. In brief, although complex interactions are involved

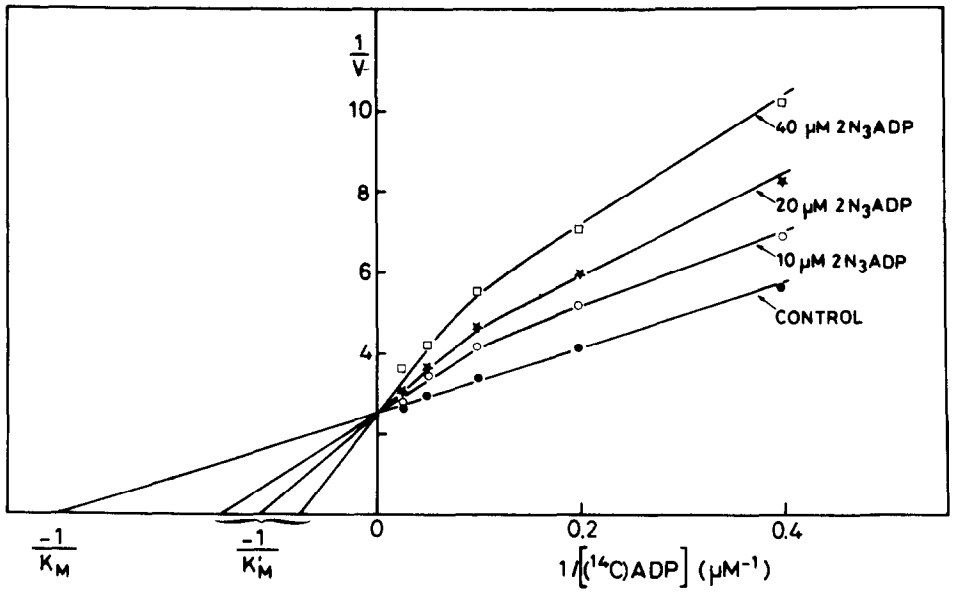

Fig.2. Inhibition of [^{14}C]ADP transport in rat heart mitochondria by 2-azido-ADP. [^{14}C]ADP transport was assayed on 1 mg of rat heart mitochondrial protein in 1 ml of the standard incubation medium in the absence or presence of different fixed concentrations of 2-azido-ADP in the dark as described in section 2.

in the inhibition of ADP transport by 2-azido ADP, a straightforward conclusion is that 2-azido ADP inhibits reversibly ADP transport probably at the level of the binding step.

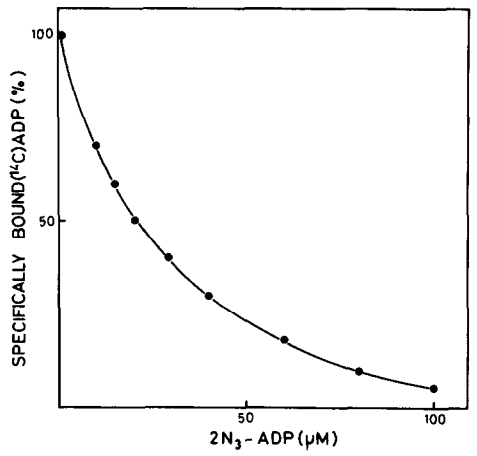

Fig.3. Release of membrane-bound [^{14}C]ADP in rat heart mitochondria upon addition of 2-azido ADP. The assay was carried out in the dark as described in section 2. The results were plotted in terms of percentages, 100% corresponding to the total amount of specifically bound [^{14}C]ADP.

3.3. *Covalent photolabeling of membrane proteins of heart mitochondria and inside-out submitochondrial particles by 2-azido-[α-^{32}P]ADP*

Because 2-azido-[α-^{32}P]ADP is not transported by the ADP/ATP carrier and is most probably non-permeant, being a charged molecule, it is expected to interact with membrane proteins directly accessible from the outside in mitochondria and submitochondrial particles. Photoirradiation of intact mitochondria with 2-azido-[α-^{32}P]ADP resulted in the photolabeling of a 32-kDa protein as shown by SDS–PAGE of a mitochondrial extract. Photoirradiation of inside-out submitochondrial particles with 2-azido-[α-^{32}P]ADP resulted in the photolabeling of the same 32-kDa protein and a 50-kDa protein (fig.4). Higher M_r compounds ($M_r > 150\,000$) revealed on the gel were probably protein aggregates. Preincubation of mitochondria with CATR, an inhibitor which binds the ADP/ATP carrier on its cytosolic face, and preincubation of inside-out submitochondrial particles with BA, an inhibitor which binds to the matrix face of the ADP/ATP carrier [19], abolished photolabeling by 2-azido-[α-^{32}P]ADP. This inhibitory effect is indicative that the photolabeled 32-kDa protein is the ADP/ATP carrier.

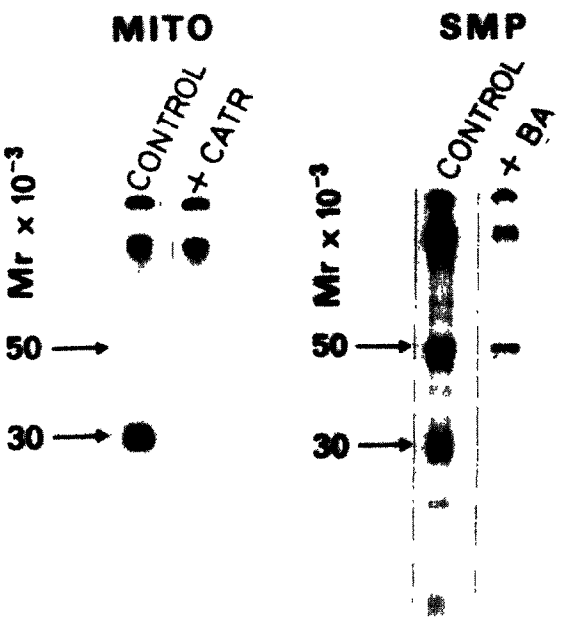

Fig.4. Photolabeleling of beef heart mitochondria and inside-out submitochondrial particles from beef heart with 2-azido-[α-^{32}P]ADP. Analysis of the photolabeled proteins by SDS-PAGE followed by fluorography. Mitochondria from beef heart and the derived sonic submitochondrial particles in the standard medium at the concentration of 1 mg protein/ml were preincubated at 0°C for 15 min with 40 μM 2-azido-[α-^{32}P]ADP and then photoirradiated as described in section 2. A parallel assay was carried out with 20 μM CATR and 10 μM BA added prior to 2-azido-[α-^{32}P]ADP, in the case of mitochondria and submitochondrial particles, respectively. After centrifugation, the pellets were lyzed by SDS and subjected to gel electrophoresis (see section 2).

The catalytic sector, F_1, of the ATPase complex in inside-out submitochondrial particles possesses nucleotide binding sites which are accessible from the external medium by added nucleotides. These sites are located on the major subunits of F_1, α and β, whose molecular mass is close to 50000. Since photolabeling of the 50-kDa protein by 2-azido-[α-^{32}P]ADP was found only in the inverted particles, a likely candidate appeared to be either the α or β subunit of F_1-ATPase. Identification of the photolabeled subunit was carried out, using isolated F_1 photolabeled by 2-azido-[α-^{32}P]ADP.

3.4. *Photolabeling of isolated F_1-ATPase by 2-azido-[α-^{32}P]ADP and identification of the photolabeled subunit*

Beef heart F_1-ATPase was preincubated in a $MgCl_2$ supplemented medium in the absence or presence of 2 mM ADP for 1 min. Then, 2-azido-[α-^{32}P]ADP was added to the final concentration of 40 μM and photoirradiation was carried out as described in section 2. The photolabeled F_1-ATPase was subjected to SDS-PAGE and to urea-PAGE to identify the labeled subunits. As a control, the α, γ and ϵ subunits were labeled by [^{14}C]NEM, and the β subunit by [^{14}C]DCCD. As illustrated in fig.5, only the β subunit was photolabeled. The specificity of the photolabeling was evidenced by the decrease of covalently bound 2-azido-[α-^{32}P]ADP resulting from preincubation of F_1 with ADP. The same specific photolabeling of the β subunit was obtained in a medium deprived of $MgCl_2$ and supplemented with 1 mM EDTA.

4. DISCUSSION

The criteria of specificity and affinity required for the binding of a photoactivable ligand to a specific receptor are fulfilled in the case of 2-azido-[α-^{32}P]ADP as summarized by the following results. (i) The reversible binding (dark assay) and the irreversible binding (light assay) of 2-azido-[α-^{32}P]ADP to the membrane-bound ADP/ATP carrier were inhibited by preincubation with two specific inhibitors of ADP/ATP transport, namely CATR and BA and with the substrate ADP. (ii) Photolabeling of F_1-ATPase by 2-azido-[α-^{32}P]ADP was inhibited by preincubation with a large excess of ADP. It should be noted, however, that 2-azido-[α-^{32}P]ADP spontaneously isomerizes into non-photoactivable tetrazole rings at neutral pH, with about 50% of the original 2-azido-[α-^{32}P]ADP being transformed at equilibrium [20]. This peculiarity introduces some complication in the assessment of the binding affinity under conditions of reversible binding (assay in the dark). However, the fact that efficient photolabeling of both the ADP/ATP carrier and F_1-ATPase is achieved at micromolar concentrations of 2-azido-[α-^{32}P]ADP and is prevented by ADP supports the idea that the photolabel binds to the ADP/ATP carrier and F_1-ATPase with high affinity.

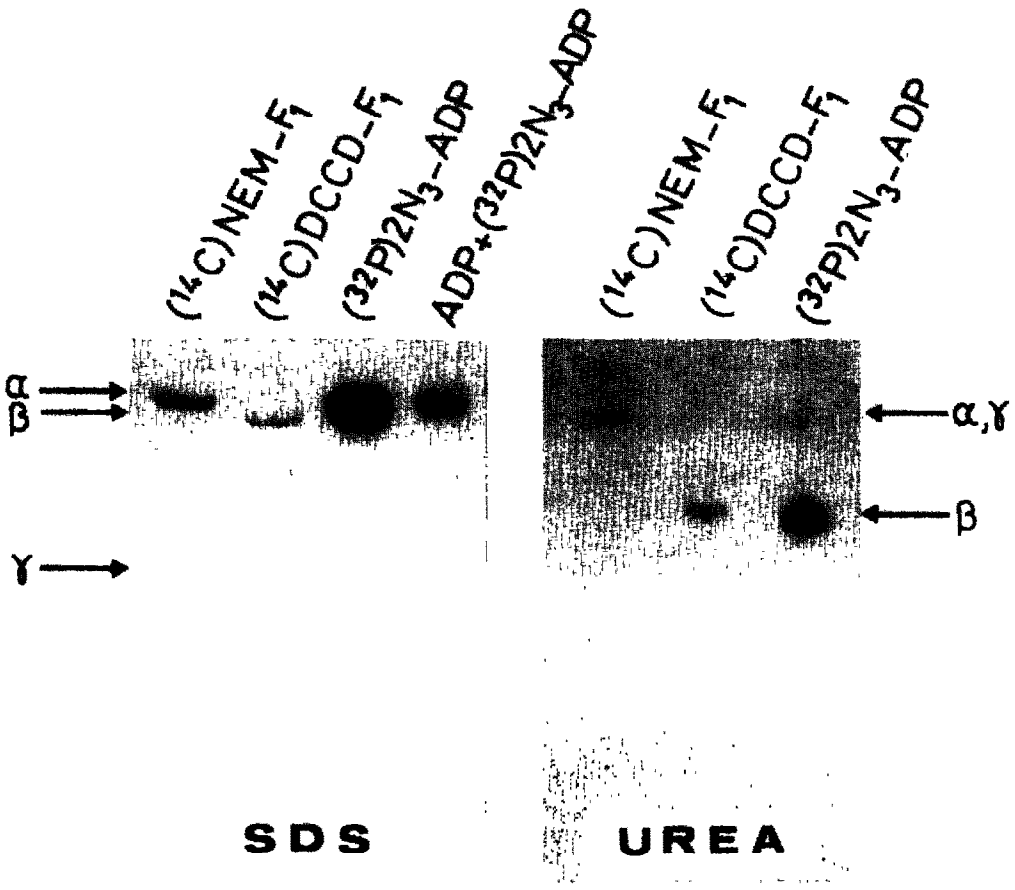

Fig.5. Photolabeling of beef heart mitochondrial F_1-ATPase with 2-azido-[α-^{32}P]ADP, and identification of the photolabeled subunits. F_1-ATPase was photoirradiated with 2-azido-[α-^{32}P]ADP, and then subjected to PAGE in the presence of SDS or urea (cf. section 2). In a parallel assay, F_1-ATPase was incubated with 2 mM ADP prior to photoirradiation with 2-azido-[α-^{32}P]ADP. The first two tracks correspond to the migration of mitochondrial F_1-ATPase subunits labeled with [^{14}C] NEM (α and γ subunits), and with [^{14}C]DCCD (β subunit). The gel electrophoresis was followed by fluorography.

The specific labeling of the β subunit of F_1-ATPase by 2-azido-[α-^{32}P]ADP is noteworthy since this subunit contains the catalytic site of the enzyme, and that other photolabeles like 8-azido ADP and 3′-arylazido ADP were found to bind to both the α and β subunits (for review cf. [21]). Clearly, 2-azido-[α-^{32}P]ADP deserves consideration for mapping studies of the adenine nucleotide binding sites in the mitochondrial ADP/ATP carrier and F_1-ATPase. An advantage of the labeling of 2-azido-ADP with ^{32}P in the α position is its stability under acidic conditions such as those used for cleavage by cyanogen bromide.

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