Mapping of Nucleotide-depleted Mitochondrial F1-ATPase with 2-Azido-[α-32P]adenosine Diphosphate

EVIDENCE FOR TWO NUCLEOTIDE BINDING SITES IN THE β SUBUNIT*

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Photolabeling of nucleotide binding sites in nucleotide-depleted mitochondrial F1 has been explored with 2-azido [α-32P]adenosine diphosphate (2-N3[α-32P]ADP). Control experiments carried out in the absence of photoradiation in a Mg2+-supplemented medium indicated the presence of one high affinity binding site and five lower affinity binding sites per F1. Similar titration curves were obtained with [3H]ADP and the photoprobe 3'-aryla azido-[3H]butyryl ADP ([3H]NAP-ADP). Photolabeling of nucleotide-depleted F1 with 2-N3[α-32P]ADP resulted in ATPase inactivation, half inactivation corresponding to 0.6–0.7 mol of photoprobe covalently bound per mol F1. Only the β subunit was photolabeled, even under conditions of high loading with 2-N3[α-32P]ADP. The identification of the sequences labeled with the photoprobe was achieved by chemical cleavage with cyanogen bromide and enzymatic cleavage by trypsin. Under conditions of low loading with 2-N3[α-32P]ADP, resulting in photolabeling of only one vacant site in F1, covalently bound radioactivity was located in a peptide fragment of the β subunit spanning Pro-320–Met-358 identical to the fragment photolabeled in native F1 (Garin, J., Boulay, F., Issartel, J.-P., Lunardi, J., and Vignais, P. V. (1986) Biochemistry 25, 4431–4437). With a heavier load of photoprobe, leading to nearly 4 mol of photoprobe covalently bound per mol F1, an additional region of the β subunit was specifically labeled, corresponding to a sequence extending from Gly-72 to Arg-83. The isolated β subunit also displayed two binding sites for 2-N3[α-32P]ADP. When F1 was first photolabeled with a low concentration of NAP-ADP, with the covalent binding of 1.5 mol of NAP-ADP/mol F1, with the bound NAP-ADP distributed equally between the α and β subunits, a subsequent photoradiation in the presence of 2-N3[α-32P]ADP resulted in covalent binding of the 2-N3[α-32P]ADP to both α and β subunits. It is concluded that each β subunit in mitochondrial F1 contains two nucleotide binding regions, one of which belongs to the β subunit per se, and the other to a subsite shared with a subsite located on a juxtaposed α subunit. Depending on the experimental conditions, the subsite located on the α subunit is either accessible or masked. Unmasking of the subsite in the three α subunits of mitochondrial F1 appears to proceed by a concerted mechanism.

The number of adenine nucleotide binding sites in the F1,1-ATPases from mitochondria, bacteria, and chloroplasts amounts to six per F1 (for review see Vignais and Satre, 1984). As there are three copies of the α subunit and three copies of the β subunit per F1, it might be inferred that these sites are equally distributed between the α and β subunits. This is in accordance with the demonstration that both the α and β subunits of F1 are covalently modified by a number of adenine-nucleotide derivatives (for review, see Vignais and Lunardi, 1985), including arylazido propionyl and butyryl derivatives (NAP-AXP and NAP-AXP) with the azido group attached by a flexible arm to the ribose moiety of the nucleotide (Lunardi et al., 1977, 1979, 1982; Conson and Guillory, 1979; Lübben et al., 1984; Fellous et al., 1984; Weber et al., 1985), dial analogs of ADP with a ribose ring opened by peroxidation (Kozlov and Milgrom, 1980; De Melo et al., 1984), a benzophenone photoactivatable derivative of ATP (Williams and Coleman, 1982), and the 8-azidoADP or -ATP (Wagendorf et al., 1979; Van Dongen et al., 1986). On the other hand, the existence of two distinct nucleotide-binding sites on isolated β subunit from Rhodospirillum rubrum F1 has recently been reported (Gromet-Elhanan and Khananshvili, 1984), as has been the prevalent labeling of two distinct regions on the β subunit of mitochondrial F1 by 2-N3ADP (Boulay et al., 1985). Not only the location but also the function of the nucleotide binding sites of F1 is controversial. It is thought that, of the six nucleotide-binding sites located on F1, three are catalytic on the basis that they exchange readily bound nucleotides during hydrolysis of ATP (Cross and Nalin, 1982). The function of the noncatalytic sites (nonexchangeable) is still disputed. Possibly, they represent regulatory sites, which would control the kinetics of ATP hydrolysis or synthesis (Di Pietro et al., 1980; Wang, 1984; Weber et al., 1985; Vasilyeva et al., 1982).

In a previous paper (Garin et al., 1986), we reported the mapping of a rapidly exchangeable site of native F1 with 2-N3[α-32P]ADP, an ADP analog which mimics the natural nucleotide (Czarnecki et al., 1982; MacFarlane et al., 1982). It was shown that photolabeling with 2-N3[α-32P]ADP was directed essentially towards the following four amino acids of the β subunit: Leu-342, Ile-344, Tyr-345, and Pro-346. Due to

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1 The abbreviations used are: F1, soluble mitochondrial ATPase from beef heart mitochondria; 2-N3ADP, 2-azido-adenosine diphosphate; NAP-ADP, 3'-aryla azido butyryl ADP; TDAB, tetradecyltrimethyl ammonium bromide; MES, 4-morpholinoethanesulfonic acid; HPLC, high performance liquid chromatography.
the apparent diversity of the nucleotide-binding sites in terms of localization and reactivity with nucleotides, it was of interest to pursue this study with the exploration of the reactivity (reversible binding in the dark and photobinding) of 2-N3[a-32P]ADP with respect to an F1 devoid of endogenous nucleotides, i.e., with all sites, in principle, accessible to added nucleotides. In the present work, we have prepared nucleotide-depleted mitochondrial F1, fully competent in catalyzing ATP hydrolysis and mapped its nucleotide-binding sites with 2-N3[a-32P]ADP under two sets of conditions. In the first one, F1 was incubated with an amount of photoprobe just sufficient to fill one nucleotide-binding site and then photolabeled. The second set of conditions consisted of loading F1 with a saturating concentration of the photoprobe added prior to photolabelling. Mapping studies showed that both conditions resulted into the labeling of essentially the β subunit. With heavy loading, a supplementary and specific binding site was demonstrated on the β subunit, clearly distinct from the first one labeled with a low concentration of the photoprobe. Unexpectedly, when F1 was prelabeled with NAP4-ADP to a limited extent, a binding region for 2-N3[a-32P]ADP on the α subunit was unmasked.

EXPERIMENTAL PROCEDURES

Materials—H3,PO4 (10 mM) was purchased from DuPont-New England Nuclear, N3[a-32P]ADP was synthesized as described by Boulay et al. (1985) with a specific activity of about 1000 dpm/pmol. Solvents used in this synthesis were redistilled. As previously mentioned (Boulay et al., 1985), 2-N3[a-32P]ADP was preferred to 2-N3[β-32P]ADP. Although its synthesis was more tedious, the probe labeled on the phosphorus group was much more stable than that labeled in the β group, which is an advantage under the drastic conditions encountered in mapping studies. [3H]NAP-ADP was synthesized by the same method as that used by Jeng and Guillory (1975) for the synthesis of unlabeled NAP-ADP. 4-[2,3-3H]Aminobutyric acid (110 Ci/mmol) was obtained from the Commissariat à l’Energie Atomique (Centre d’Etudes Nucléaires, Saclay, France) and diluted before use with unlabeled 4-aminobutyric acid. The specific activity of [3H]NAP-ADP ranged between 300 and 400 dpm/pmol.

Beef heart mitochondria were prepared as described by Smith (1967) and used for the preparation of F1-ATPase according to the method of Knowles and Penefsky (1972) with some modifications introduced by Klein et al. (1979). Nucleotide-depleted F1 was prepared as described by Issartel et al. (1983). In brief, F1 was dissociated into its subunits by incubation in a medium consisting of 50 mM succinate-Tris, 1 M NaCl, 0.25 M NaNO3, 0.1 mM dithiothreitol, and 4 mM EDTA, final pH 6.1. The fraction was dialyzed overnight against the same medium and frozen at –80°C for 24 h. It was then thawed and dialyzed again against another medium consisting of 25 mM succinate, 4 mM EDTA, 5 mM ATP, and 2 mM 2-mercaptoethanol, final pH 6.5 (ATP medium). Finally, it was chromatographed on a DE52 cellulose (Whatman) column equilibrated with the ATP medium, using a gradient of LiCl.

Two main peaks were recovered, the first one corresponding to the αβ complex and the second one to the β subunit. Before use, the β subunit was desalted by passage through a Sephadex G50 column equilibrated with the reaction medium (Penefsky, 1977).

Biological Assays—The ATPase activity of nucleotide-depleted F1, was tested with regenerating medium (Pullman et al., 1960). Its value (75–85 μmol/min/mg protein at 30°C) was virtually identical to that of the ATPase activity of the native enzyme. Radioactivity was measured by liquid scintillation counting (Patterson and Greene, 1965). The protein concentration was estimated as described by Bradford (1976). A molecular weight of 371,000 was used for stoichiometry determinations (Walker et al., 1985). For measurement of binding, F1 was heated at 95°C for 2.5 min (Issartel et al., 1986), and the extracted nucleotides were assayed by a luminescence test (Lundin et al., 1976).

Polyacrylamide Gel Electrophoresis—Routine gel electrophoresis of F1 was run on 12% sodium dodecyl sulfate-polyacrylamide slab gels according to the method of Laemmli (1970). When better resolution between the α and β subunits was needed, sodium dodecyl sulfate was replaced by TDA, a cationic detergent (Amory et al., 1980). For polypeptide mapping, the polyacrylamide gel was stained with 0.5% linear polyacrylamide to prevent cracking of the gels during drying. The buffers were prepared as described by Cabral and Schatz (1979).

Reversibly Binding of Radiolabeled Nucleotides—Samples of nucleotide-depleted F1 were incubated to a final concentration of about 1 μM in 1 M NaCl at 25°C with increasing concentrations of 2-N3[a-32P]ADP, [3H]NAP-ADP, or [14C]ADP in 0.1 ml of a reaction medium containing 100 mM sucrose, 30 mM Tris-HCl, 50 mM NaCl, 3 mM MgCl2, and 7.5% glycerol (w/v), final pH 7.5. After a 60-min incubation, the samples were filtered by centrifugation through Sephadex G50 (fine) contained in tuberculin syringes of 1 ml volume inserted in conical centrifuge tubes according to the method described by Penefsky (1977), recently modified to avoid loss of the titrated nucleotides. In brief, the amounts of wet Sephadex G50 in the tuberculin syringes was decreased to 0.8 ml for 80 μl of the F1 solution corresponding to 20–30 μg of protein, and the centrifugation of the proteins was increased to 450 × g at the top of the gel for 20 s.

Photoactivation Assays—Nucleotide-depleted F1 was dialyzed for 1 h against 1 liter of a buffer containing 50 mM Tris base, 50 mM MES, 2 mM MgCl2, final pH 7.3 (TMM buffer). In routine assays, dialysis buffer containing [3H]NAP-ADP, [14C]ADP, or [14C]ADP in the dark at 25°C for 30 min prior to photoirradiation with a Xenon lamp XBO 1000 W/HS equipped with a parabolic reflector and placed at 30 cm from the sample (Muller Gmb, Moosinzing, West Germany). The concentration of the photoprobe and the period of irradiation used depended on the extent of photolabeling that was desired. In some experiments, F1 was subjected to several cycles of photoactivation. Control experiments showed that less than 5% of ATPase activity was lost during photoirradiation in the absence of photoprobe. Photoirradiation in the presence of [3H]NAP-ADP or 2-N3[a-32P]ADP was followed by incubation with 5 mM ADP for 15 min at 30°C to displace the noncovalently bound azido nucleotides. The covalently modified F1 was finally recovered in the excluded fraction after elution centrifugation through Sephadex G50 columns, using the technique described by Penefsky (1977). It was finally assayed for radioactivity, protein content, and ATPase activity.

Processing of the Photolabeled β Subunit—The photolabeled β subunit in nucleotide-depleted F1, was obtained after dissociation of F1 into subunits as described above and freeze-dried. It was solubilized in 7 M guanidinium chloride, pH 9.0, at a concentration of 5 mg/ml. Succinylation of the amino groups was performed as described by Walker et al. (1980). Further processing of the succinylated β subunit, digestion with trypsin, and characterization by peptide mapping and amino acid analysis were performed by the method of Issartel et al. (1983). Nucleotide-depleted F1 was incubated with 5 mM ADP for 15 min at 30°C to display the noncovalently bound azido nucleotides. The covalently modified F1 was finally recovered in the excluded fraction after elution centrifugation through Sephadex G50 columns, using the technique described by Penefsky (1977). It was finally assayed for radioactivity, protein content, and ATPase activity.

Amino Acid Composition—Peptide hydrolysis was performed in a sealed tube with 150 μl of HCl containing 0.005% (w/v) phenol at 110°C for 20 h. After hydrolysis, the content of the tube was dried under vacuum, and the residue was taken up in 0.06 M sodium citrate buffer, pH 3.1. The amino acids were post derivatized with O-phthalaldehyde. Amino acid analysis was performed by means of a Catex resin column (Waters) following the instructions of the manufacturer, and the derivatized amino acids were assayed at 420 nm with the fluorescence detector.

Identification of Amino-terminal Amino Acids—The peptide to analyze (0.1 nmol) was dissolved in 25 μl of 0.2 M sodium phosphate buffer, pH 7.0, and mixed with 1 μl of a freshly prepared aqueous solution of amino peptidase M (Boehringer Mannheim) at 0.25 mg/ml.

J. Lunardi, manuscript in preparation.
RESULTS

The Six Nucleotide-binding Sites of Nucleotide-depleted Mitochondrial F1 Can Be Filled with 2-N3[γ-32P]ADP: Comparative Studies with ADP and NAP₄-ADP—The nucleotide-depleted F1 preparations used for the present work contained an average of 0.3–0.5 mol of ADP and ATP/mol F1. The ability of depleted F1 to bind nucleotides in the presence of MgCl₂ was assayed with the natural nucleotide ADP and two azido derivatives of ADP known to inactivate and photolabel F1 upon photoirradiation, namely 2-N₃ADP and NAP₄-ADP. Equilibrium between bound and free nucleotide was attained in less than 10 min at room temperature, a time period which was much shorter than the incubation period of 30 min used in most of the binding experiments (data not shown). The assays for the reversible binding of 2-N₃[γ³²P]ADP and [³H]NAP₄-ADP were carried out in dim light to avoid photoactivation of the probe.

The data in Fig. 1 illustrate the binding of 2-N₃[γ³²P]ADP. The corresponding Scatchard plots were curvilinear, indicating the presence of heterogeneous binding sites. Similar binding curves were obtained for [¹⁴C]ADP and [³H]NAP₄-ADP. In brief, there are two possible alternatives to explain these curvilinear plots on the basis of a multisite model: 1) the different classes of sites do not interact and differ by their Kd values, Kd₁, Kd₂, Kd₃, etc. In this case, the number of bound nucleotides Nₚ at a given concentration of free nucleotide S is related to the total number of sites, N, and the Kd values by the equation:

\[ Nₚ = N \left( \frac{S}{Kₐ₁ + S} + \frac{S}{Kₐ₂ + S} + \frac{S}{Kₐ₃ + S} + \ldots \right) \]

2) The sites are equivalent in the absence of nucleotide; upon addition of nucleotide, titration of the first available site by a nucleotide lowers the binding affinity of the other sites.

Because the differences in Kd values were not large enough, we preferred to refer to two classes of sites with Kd values lower than 0.5 μM for high affinity sites and Kd values ranging between 1 and 8 μM for low affinity sites (Table 1). Of the six nucleotide-binding sites titrated with ADP, 2-N₃ADP and NAP₄-ADP, one exhibited a high affinity binding for the three nucleotides with a Kd of 0.1–0.4 μM and the other five a lower affinity with a Kd of 1–8 μM.

Photoirradiation of Nucleotide-depleted F1 by 2-N₃[γ³²P]ADP Results in Photolabeling of Only the β Subunit—Photolabeling of nucleotide-depleted F1 by 2-N₃[γ³²P]ADP was accompanied by inactivation of F₁: ATPase. Under similar conditions of photoirradiation in the absence of photoprobe, there was no significant loss of ATPase activity. The extent of covalent photolabeling increased with the concentration of 2-N₃[γ³²P]ADP. It was checked that photolabeling of F₁ by 2-N₃[γ³²P]ADP at concentrations lower than 150 μM was prevented by about 95% by preincubation with 10 mM ADP, pointing to the specific labeling of nucleotide-binding sites.

To remove the noncovalently bound analog 5 mM ADP was added after photoirradiation and incubated with the photolabeled F₁ for 15 min, followed by centrifugation filtration on a Sephadex G50 column. The ATPase activity and the radioactivity of the eluate were measured. To assess the percentage of inactivation, a control assay was performed in the absence of the photoprobe, but in the presence of 5 mM ADP and under photoirradiation. Maximal inactivation in this control was below 5%. As shown in Fig. 2, a linear relationship between the amount of covalently bound 2-N₃[γ³²P]ADP and the extent of inactivation was found up to 60% inactivation, with half inactivation corresponding to 0.6–0.7 mol of 2-N₃[γ³²P]ADP bound/mol of depleted F₁. Beyond 60% inactivation, the plots departed from linearity. The initial linear portion of the plots extrapolated to 100% inactivation intercepted the abscissa at a value of 1.4 mol of bound photoprobe/mol of F₁. However, full inactivation required up to 3 mol of bound photoprobe/mol F₁. Addition of 5 mM ADP prior to that of 2-N₃[γ³²P]ADP and photoirradiation prevented photolabeling and photoinactivation of F₁, pointing to the specific recognition of the photoprobe by nucleotide-binding sites.

Even when the concentration of 2-N₃[γ³²P]ADP used during the preincubation period in the dark was in large excess with respect to that of F₁, leading to the reversible binding of 5–6 mol of the photoprobe/mol F₁, more than 90% of the covalently bound 2-N₃[γ³²P]ADP after photoirradiation was located on the β subunit (Fig. 3).

More Than One Site Per Isolated β Subunit Is Photolabeled with 2-N₃[γ³²P]ADP—ADP and ATP bind to the isolated β subunit with a much lower affinity than to the β subunit integrated in the F₁ structure. However, specific ADP-
TABLE I

| Binding parameters of 2-N$_2$[a-32P]ADP, $^3$HJADP, and $^3$H]NAP$_2$-ADP to nucleotide-depleted F$_1$
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | $N_1$ | $K_d$ | $N_2$ | $K_d$
| ADP              | 1     | 0.1   | 4–5   | 1–2   |
| 2-N$_2$ADP       | 1     | 0.3   | 4–5   | 3–8   |
| NAP$_2$-ADP      | 1     | 0.4   | 4–5   | 5–7   |

FIG. 2. Correlation between covalent photolabeling and photoinactivation of nucleotide-depleted F$_1$ by 2-N$_2$[a-32P] ADP, F$_1$ (35 µg) in 0.1 ml of 100 mM sucrose, 30 mM Tris-Cl, 50 mM NaCl, 3 mM MgCl$_2$, and 7.5% glycerol, final pH 7.5, was preincubated for 30 min in the dark in the presence of increasing concentrations of 2-N$_2$[a-32P]ADP, ranging between 0.1 and 60 µM and then photoirradiated twice, for 3 s each time, as described under "Experimental Procedures." The noncovalently bound photoprobe was displaced by ADP and removed by gel filtration (cf. "Experimental Procedures"). ATPase activity and covalent binding of the photoprobe were determined. Control ATPase activity was assayed with F$_1$ samples photoirradiated under the same conditions as above, except that the photoprobe was omitted.

ATP-binding sites could be revealed in the presence of aurovertin (Lunardi et al., 1986). Photolabeling with 2-N$_2$[a-32P] ADP also required a much higher concentration of the probe than photolabeling of F$_1$ did. To assess the specific photolabeling directed to the nucleotide-binding sites, parallel assays were performed with 10 mM ADP added prior to 2-N$_2$[a-32P] ADP. Specific labeling corresponded to about 85% of the total photolabeling. Photoirradiation of isoiotd $\beta$ subunit with increasing concentrations of 2-N$_2$[a-32P]ADP resulted in a steady increase of the specific covalent binding of the photoprobe up to 200 µM, where the titration curve showed an inflexion. At 300 µM 2-N$_2$[a-32P] ADP, the amount of specifically bound probe was 1.2 mol/mol F$_1$ (Fig. 4).

As reported by Czarnecki (1984), at neutral pH about 50% of the 2-N$_2$[a-32P] ADP is under a tetrazolo form which is still able to recognize the nucleotide-binding sites of F$_1$ as efficiently as the nitreno form, but is unable to bind covalently to these sites upon photoirradiation (Boulay et al., 1985). This is expected to lower substantially the yield of photolabeling. The binding data of Fig. 4 strongly suggest, therefore, that each $\beta$ subunit contains two nucleotide-binding sites.

Identification of Two Photolabeled Peptide Sequences in the $\beta$ Subunit of Nucleotide-depleted F$_1$—First, it was decided to check whether the peptide sequence corresponding to the nucleotide-binding site that was filled at low concentrations of 2-N$_2$[a-32P] ADP was the same in native F$_1$ and in nucleotide-depleted F$_1$. Native F$_1$ still contains three tightly bound nucleotides (Garrett and Penefsky, 1975). Nucleotide-depleted F$_1$ and native F$_1$ were photolabeled with 2-N$_2$[a-32P] ADP added at a concentration just sufficient to fill one vacant site. The photolabeled $\beta$ subunits of the two F$_1$ preparations, native F$_1$, and nucleotide-depleted F$_1$ were isolated and
bound photoprobe. They were then subjected to centrifugation filtration through Sephadex G50 (fine). Control assays were run with the samples were photoirradiated twice

cova lent binding. The curve illustrated in Fig. 4 corresponds to the specific covalent binding of 2-Ns[α-32P]ADP (prevented by ADP). Inset, autoradiography of a gel after electrophoresis of photolabeled β subunit without ADP (gel 2) and with ADP (gel 3) added prior to 3-N3ADP. Gel 1 was colored by Coomassie Blue.

cleaved at methionyl residues with CNBr under similar conditions. The cleavage products were separated on a cellulose thin layer plate by electrophoresis followed by chromatography (cf. “Experimental Procedures”). The autoradiography revealed the same radioactive peptide maps for native and nucleotide-depleted F1 (Fig. 5). One of the two major radioactive peptides corresponded to a sequence already identified in native F1, as that spanning residues Gln-293-Met-358 that the radioactive material of peak GII corresponded to CB9. The small radioactive peak at the level of GIII was identified to CB’9 (see above).

The second nucleotide-binding site located in the β subunit and photolabeled with 2-Ns[α-32P]ADP at high concentration was explored with nucleotide-depleted F1 as this site was more accessible and reactive to the photoprobe in nucleotide-depleted F1 than in native F1. Photoirradiation was carried out with 2-Ns[α-32P]ADP added by increments with several cycles of photoirradiation until about four sites/F1 were covalently photolabeled. They were then subjected to centrifugation filtration through Sephadex G50 (fine). Control assays were run with the samples were photoirradiated twice

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The present work, dealing with nucleotide-depleted F1 photolabeled by 2-Ns[α-32P]ADP at low concentration, the same criteria were used and led to the same conclusion, namely that the radioactive material of peak GII corresponded to CB9. The small radioactive peak at the level of GIII was identified to CB’9 (see above).

An alternative method, previously described for native photolabeled F1 (Garin et al., 1986), was used to map the nucleotide-binding site of the β subunit of nucleotide-depleted F1 photolabeled at low concentrations of 2-Ns[α-32P]ADP. The β subunit obtained from dissociated photolabeled F1 was succinylated and subjected to cleavage by CNBr. The cleavage products were chromatographed on Sephadex G75. The radioactivity profile resembled that described in the previous paper dealing with native F1 (Garin et al., 1986) with one major radioactive peak, GII, and two minor radioactive ones, GI and GIII (Fig. 6, inset). On the basis of the amino acid composition and the amino-terminal amino acid, Garin et al. (1986) had assigned the radioactive peptide present in peak GII to fragment CB9 in the nomenclature of Runswick and Walker (1983). In the present work, dealing with nucleotide-depleted F1 photolabeled by 2-Ns[α-32P]ADP at low concentration, the same criteria were used and led to the same conclusion, namely that the radioactive material of peak GII corresponded to CB9. The small radioactive peak at the level of GIII was identified to CB’9 (see above).
succinylation, cleavage by CNBr, and fractionation by filtration on Sephadex G75. The same three peaks of radioactivity (designated by GI, GII, and GIII) as those mentioned above (inset of Fig. 6) were resolved (Fig. 6). They contained 10, 49, and 41% of the bound radioactivity, respectively. As reported by Runswick and Walker (1983), peak GIII contains the CB2, CB3, CB5, CB6, CB10, and CB12 peptides, and in our hands CB9, the fragmentation product of CB9 mentioned above. In comparison to peak GIII obtained from F1 photolabeled at one vacant site (inset of Fig. 6), peak GIII obtained from F1 photolabeled at four sites (Fig. 6) contained a higher amount of bound radioactivity which was differently distributed. Instead of a small peak of radioactivity, there was a large radioactive peak (designated by arrow 2) with a shoulder (designated by arrow 1). The shoulder corresponded to radioactive CB9 peptide.

For further identification, the material corresponding to the maximum of radioactivity (arrow 2) in peak GIIII in trypsinized (cf. "Experimental Procedures")), and the digest was fractionated by Bio-gel P-4 (Bio-Rad) chromatography (Fig. 7, panel A). The elution pattern showed two main peaks of absorbancy. More than 90% of the radioactivity was located in the second peak corresponding to fractions 34–39 which contained trypsinized peptides originating from CB9, CB5, CB3, and CB10. These fractions were pooled, concentrated, and subjected to HPLC using a gradient of acetonitrile (Fig. 7, panel B). By this procedure, a radioactive peptide was isolated, which was clearly different from the tryptic cleavage products of the CB9 peptide also present in peak GIIII. Its amino acid composition was determined (Table II). It was essentially identical to that of the tryptic peptide R9 spanning Gly–72–Arg–83 in the β subunit (Runswick and Walker, 1983). The lack of lysine residues in the radioactive peptide could be explained by the fact that the β subunit was succinylated. To ascertain the identity of this photolabeled peptide, the amino-terminal sequence was analyzed after digestion with amino-peptidase M. Hydrolysis for 1 and 2 h under conditions described under "Experimental Procedures" yielded glycine and glutamine in ratios of 2:1.4, respectively. This indicated a Gly–Gln amino-terminal sequence, in full agreement with the known amino-terminal sequence of peptide R9.

In conclusion, when F1 is photoirradiated in the presence of an excess of 2-N3[α-32P]ADP, an additional region of the β subunit becomes photolabeled. It corresponds to peptide R9 spanning Gly–72–Arg–83.

The Sequential Photoirradiation of Nucleotide-depleted F1 by NAP4-ADP and 2-N3[α-32P]ADP Results in the Covalent Labeling of Both the α and β Subunits by 2-N3[α-32P]ADP—It has been previously reported that photoirradiation of native mitochondrial F1 with [3H]NAP4-ADP results in the covalent labeling of the α and β subunits and that full inactivation of F1 corresponds to the covalent binding of 2 mol of photoprobe/mol of F1, one on the α subunit and the other on the β subunit (Lunardi and Vignais, 1979; Cosson and Guillory, 1979). In view of the nearly exclusive photolabeling of the β subunit of F1 by 2-N3[α-32P]ADP, the nature of the covalent photolabeling of the α subunit by NAP4-ADP could be questioned. Did the labeling of the α subunit reflect the presence of a nucleotide-binding site on the α subunit or was it a consequence of the geometry of the probe, with the azido group attached to the ribose moiety of ADP through a flexible and mobile arm capable of interacting with a region of the α subunit in close contact with β, but devoid of a nucleotide-binding site?

The experiments described hereafter point to specific nucleotide-binding sites on the α subunit. Nucleotide-depleted F1 was photolabeled to various extents by photoirradiation with increasing concentrations of [3H]NAP4-ADP in the presence of MgCl2. After removing the noncovalently bound [3H] NAP4-ADP, photolabeled F1 was incubated in the dark with 100 μM 2-N3[α-32P]ADP for 30 min, followed by centrifugation and filtration on Sephadex G50 (cf. "Experimental Procedures"). The concentration of 2-N3[α-32P]ADP used was high enough to saturate all vacant nucleotide-binding sites on F1. Despite the large variation in the amounts of covalently bound [3H] NAP4-ADP and reversibly bound 2-N3[α-32P]ADP, the sum of the two bound photoprobes remained constant and close to 5 mol of bound nucleotide/mol of F1 (Table III). Mutual exclusion of 2-N3[α-32P]ADP by [3H]NAP4-ADP for binding to F1 suggested either common binding sites for both nucleotides or selective site-site interactions.

In another experiment, nucleotide-depleted F1 was first photoirradiated with [3H]NAP4-ADP, under conditions of covalent binding of 1.6 mol of [3H]NAP4-ADP/mol of F1. On a fraction, it was checked by TADB-polyacrylamide gel electrophoresis and analysis of radioactivity in slices of the gel that 1H radioactivity was distributed equally between the α and β subunits. After removing the noncovalently bound [3H] NAP4-ADP by washing with unlabeled ADP as described above, F1 was further photolabeled with 100 μM 2-N3[α-32P]ADP, resulting in the covalent binding of 2.2 mol of 2-N3[α-32P]ADP/mol of depleted F1. The specific radioactivity of [3H] NAP4-ADP was markedly less than that of 2-N3[α-32P]ADP so that, in the later stage of the experiment dealing with gel autoradiography, only the 32P radioactivity was detected. The

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**Fig. 6.** Distribution of 32P radioactivity in the CNBr cleavage products from nucleotide-depleted F1, photolabeled with 2-N3[α-32P]ADP under conditions of high loading with the photoprobe. Nucleotide-depleted F1 (6.5 μM) was incubated with 2-N3[α-32P]ADP under conditions leading to the occupancy of more than five sites (cf. Fig. 1) and then photoirradiated. Three cycles of photoirradiation of 10 s each were performed, leading to the covalent binding of about 4 mol of photoprobe/mol of F1. The photolabeled β subunit was isolated and cleaved by CNBr as described under "Experimental Procedures." The cleavage products corresponding to 6 mg of protein were applied to a Sephadex G75 superfine column (120 × 2 cm) in 50 mM ammonium bicarbonate. Fractions of 1.75 ml were collected. The recovery of the radioactive material was 85%. The bars designate the GI, GII, GIII pooled fractions. Arrow 1 in peak GIII corresponds to the radioactive CB9 peptide and arrow 2 to an additional labeled peptide (see Fig. 7). Inset, same photolabeling experiment with nucleotide-depleted F1, as above, except photolabeling was carried out under conditions of low loading with the photoprobe, leading to the occupancy of about one site before photoirradiation. Arrow 1 corresponds to radioactive CB9.
Mapping of Nucleotide-binding Sites in $F_1$

Amino acid composition of the 2-N$_3$[α-32P]ADP-labeled tryptic peptide isolated from the GII1 fraction

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>2-N$_3$[α-32P]ADP-labeled peptide$^a$</th>
<th>R$_e$ peptide$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td>Glu</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>Succinylated</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Asp</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Ser</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td>Ala</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Pro</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Ile</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ Number of amino acid residues experimentally determined and normalized on the basis of 1 Arg residue.

Nucleotide-depleted $F_1$ (4 μM) was photoradiated first with increasing concentrations of [3H]NAP4-ADP for covalent photolabeling. After filtration on Sephadex G50, the sample was incubated in the dark with 100 μM 2-N$_3$[α-32P]ADP for 30 min. After an additional gel filtration, the amounts of bound [3H]NAP4-ADP and 2-N$_3$[α-32P]ADP were determined.

<table>
<thead>
<tr>
<th>Bound to $F_1$</th>
<th>Bound to $F_1$ and $F_2$</th>
<th>Sum of bound [3H]NAP4-ADP and 2-N$_3$[α-32P]ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]NAP4-ADP</td>
<td>2-N$_3$[α-32P]ADP</td>
<td>2-N$_3$[α-32P]ADP</td>
</tr>
<tr>
<td>mol/mol $F_1$</td>
<td>mol/mol $F_1$</td>
<td>mol/mol $F_1$</td>
</tr>
<tr>
<td>none</td>
<td>4.30</td>
<td>4.30</td>
</tr>
<tr>
<td>0.83</td>
<td>3.49</td>
<td>4.32</td>
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<td>1.80</td>
<td>2.59</td>
<td>4.39</td>
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<td>3.47</td>
<td>1.29</td>
<td>4.76</td>
</tr>
<tr>
<td>4.25</td>
<td>0.63</td>
<td>4.88</td>
</tr>
</tbody>
</table>

The doubly photolabeled $F_1$ was subjected to TDAB-polyacrylamide gel electrophoresis, followed by autoradiography. Surprisingly, 32P radioactivity was found in both the α and β subunits (Fig. 8, lane 2). In the experiment illustrated in Fig. 8, the amount of 2-N$_3$[α-32P]ADP incorporated into the α subunit was about 10% of the total covalently bound probe.

DISCUSSION

Earlier photolabeling studies carried out with 2-N$_3$[α-32P] ADP on native mitochondrial $F_1$, i.e. $F_1$, still containing tightly bound adenine nucleotide, led to the conclusion that two different nucleotide-binding sites located on the β subunit were photolabeled (Boulay et al., 1985). The two sites differed in their reactivity to the photoprobe, one of them being much more photolabeled than the other. Evidence that these two sites corresponded to different regions of the α subunit accessible to 2-N$_3$[α-32P]ADP. Most likely this region is close to, and even at the interface with, the β subunit.
Mapping of Nucleotide-binding Sites in F1

Binding Properties of Nucleotide-depleted Mitochondrial F1.—The contention that the number of nucleotide-binding sites per F1 amounts to six is based on the sequential filling of vacant sites in nucleotide-depleted F1, with a variety of nucleotides (Cross and Nalin, 1982; Weber et al., 1985). In the present work nucleotide-depleted F1 was also used, and conditions are described that lead to the reversible binding (in the absence of photolabeling) of nearly 6 mol of 2-N3-ADP of F1. In other words, full loading of the three catalytic sites and the three noncatalytic sites was achieved with a single species of nucleotide. As the specificity of the noncatalytic sites of F1 is narrow (Schuster et al., 1975; Harris, 1978), it is concluded that 2-N3-ADP mimicks ADP for recognition of the noncatalytic sites. For 2-N3-ADP as for ADP, the six binding sites fell in two classes, one high affinity class consisting of essentially one high affinity site (Kd < 0.5 μM) and five lower affinity sites (Kd > 1 μM). The high affinity binding site is probably the same as that titrated in the native enzyme, belonging to the class of exchangeable sites (Lunardi et al., 1986). The present results are in contrast with those reported by Tiedge et al. (1982) who found in nucleotide-depleted F1, only three titratable ADP binding sites, namely one high affinity site with a Kd of 50 nM and two lower affinity sites with a Kd of 3 μM. Presumably, these differences depend on the F1 preparations or the experimental conditions used for nucleotide binding.

Differential reactivity of the binding sites with respect to 2-N3[32P]ADP was evidenced by the results of the photo-labeling experiments. Heavy nucleotide loading and several cycles of photolabeling led to the preferential labeling of the catalytic sites with 75% of covalently bound 2-N3-ADP located in the CB9 sequence. The remaining bound 2-N3-ADP was located in the R6 sequence which is presumed to be part of the noncatalytic site, probably a tight, nonreadily exchangeable site or in its close neighborhood.

Because of the one-third site reactivity of F1, as illustrated by inactivation with a number of chemical modifiers and photoprobes (for review, see Vignais and Lunardi, 1985), it
was expected that covalent photolabeling of the high affinity nucleotide-binding site by 2-N3-ADP would result in full inactivation. As shown by the data in Fig. 2, this is not the case. A simple explanation for the nonlinear relationship between photolabeling and inactivation is that F1 is able to hydrolyze ATP with different turnovers depending on the availability of three, two, or one catalytic sites. In other words, with three or two sites, catalysis would be accomplished by the alternate site mechanism (Boyer et al., 1977); a higher rate of catalysis is postulated with three sites. When two sites are modified, ATP hydrolysis proceeds at a much slower rate by unisite catalysis. Consequently, the first rectilinear portion of the correlation curve (up to 60% inactivation) would reflect the preferential photolabeling of one high affinity site on one β subunit in the overall population of F1 molecules. The curve found above 90% inactivation would reflect the random binding of two or three photoprobes to the remaining population of F1 molecules. Another possible explanation is that, upon covalent binding of one 2-N3-ADP to one β subunit of F1, a change of conformation immediately occurs, which makes a second β subunit much more reactive to the photoprobe. This would be consistent with the fact that, in the correlation curve, extrapolation of the initial rectilinear portion to 100% inactivation corresponds to the binding of more than 1 mol of 2-N3-ADP/mol of F1.

Evidence for Two Nucleotide-binding Sites Per β Subunit—There are several pieces of evidence arising from the present work in favor of two nucleotide-binding sites per β subunit. (a) More than 1 mol of 2-N3[α-32P]ADP binds covalently to 1 mol of isolated β subunit upon photoirradiation. (b) Two sets of amino acid residues in the β subunit are photolabeled when nucleotide-depleted F1 is photoprotected in the presence of 2-N3[α,β-33P]ADP. (c) Essentially the β subunit in F1 is photolabeled by 2-N3[α,β-33P]ADP, even in a situation where 5-6 mol of probe are bound per mol of F1, in the dark and where, upon photoproteinization, the covalently bound probe amounts up to 4 mol/mol F1.

Gromet-Elhanan and Khananshvili (1984) have reported the presence of two nucleotide-binding sites per β subunit in F1, isolated from chromatophores of R. rubrum, on the basis of direct titration data. Boulay et al. (1985) have also presented evidence for two nucleotide-binding sites in the β subunit of mitochondrial F1, from mapping studies with the V8 protease of S. aureus (Boulay et al., 1985). At first sight, these two conclusions are in contradiction with those derived from the use of other photoprobes, namely the 8-azido adenine nucleotides (Wagenvoord et al., 1979), the arylazido nucleotides where the substituted residue is the 3' OH group of the ribose moiety (Russel et al., 1976; Lunardi et al., 1977, 1979; Tiedje et al., 1982), and the benzophenone derivative of ATP (Williams and Coleman, 1982). In all these cases, both the α and β subunits of F1 were labeled, leading to the conclusion that the six nucleotide-binding sites of F1 are equally distributed between the α and β subunits. There is no explanation for the positive reactivity of the α subunit of mitochondrial F1, to the arylazido nucleotides and the benzophenone derivative of ATP and its absence of reactivity to 2-N3ADP. It is, however, possible that the arylazido and benzophenone moieties of the above analogs, because of their lipophilic nature, have access to a hydrophobic pocket of the α subunit which contains part of a nucleotide-binding site.

An unexpected finding was that partial photolabeling of nucleotide-depleted F1, with NAP-1,ADP on one α and one β subunit allows the covalent binding of 2-N3[α,β-33P]ADP to the other α subunits which were otherwise inaccessible. This finding opens the possibility of a masked subsite on the α subunit of F1, which is unmasked and thereby made accessible to 2-N3-ADP under appropriate conditions. A reasonable explanation is that each β subunit contains two nucleotide-binding regions, one of which is a site contained within the β subunit, whereas the other is a subsite shared with another subsite on a juxtaposed α subunit. In this context, it is possible that photolabeling of the α subsite arises at the expense of the β subsite by motion of the adenine ring between the α and β subunits. Under the experimental conditions described in this paper, at most, one α subunit and one β subunit per F1 were photolabeled by NAP-1,ADP. It is likely that the binding of NAP-1,ADP to the nucleotide-binding region on one α subunit results in the unmasking of the same region on the other two α subunits by a concerted mechanism of conformational change. One may wonder whether, in the course of catalysis, the adenine ring of the nucleotide located at the αβ interface does not move between the α and β subunits as proposed for the 2-azido adenine ring (see above). If this were the case, the three sites at the αβ interfaces would have the possibility of indirectly controlling the catalytic efficiency of F1.

The idea of a shared site in mitochondrial F1 has already been suggested (Cossen et al., 1979; Lunardi and Vignais, 1982; Williams and Coleman, 1982; Lubben et al., 1984; Boulay et al., 1985; Van Dongen and Beden, 1986). From the data obtained with native F1, the hypothesis was made (Boulay et al., 1985) that, of the six nucleotide-binding sites per F1, the three exchangeable sites belong to the β subunits per se and the three nonreadily exchangeable sites are shared by α and β subunits. Because of the similarities in photolabeling of native F1 and nucleotide-depleted F1, it may be inferred that the readily photolabeled size in nucleotide-depleted F1 is one of the three exchangeable sites in native F1, i.e. a catalytic site. At the readily exchangeable site, 2-N3[α,β-33P]ADP is known to bind covalently to Leu-342, Ile-344, Tyr-345, and Pro-346. Interestingly, Tyr-345 is also photolabeled by [1H] p-fluorosulfonyl-benzoyl-5'-inosine, an affinity analog of ITP (Bullough and Allison, 1986). As ITP is thought to react only with the catalytic site of F1, (Schuster et al., 1975), it is inferred that Tyr-345 is part of the catalytic site of the β subunit, which is in agreement with the contention that the readily exchangeable site to which 2-N3[α,β-33P]ADP binds is also the catalytic site. It is noteworthy that a different distribution of nucleotide-binding sites is encountered in the case of F1, from Escherichia coli and thermophilic bacterium PS5, where the presence of two distinct sites unique to the α and β subunits has been demonstrated (Dunn and Futai, 1980; Ohga et al., 1980; Issartel and Vignais, 1984; Hisabori et al., 1986; Röger et al., 1986).

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