Identification of Amino Acid Residues Photolabeled with 2-Azido[α-^{32}P]adenosine Diphosphate in the β Subunit of Beef Heart Mitochondrial F_1-ATPase†

Jérome Garin,* François Boulay, Jean Paul Issartel, Joël Lunardi, and Pierre V. Vignais

Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France

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ABSTRACT: When beef heart mitochondrial F_1-ATPase is photoirradiated in the presence of 2-azido[α-^{32}P]adenosine diphosphate, the β subunit of the enzyme is preferentially photolabeled [Dalbon, P., Boulay, F., & Vignais, P. V. (1985) *FEBS Lett. 180*, 212–218]. The site of photolabeling of the β subunit has been explored. After cyanogen bromide cleavage of the photolabeled β subunit, only the peptide fragment extending from Gln-293 to Met-358 was found to be labeled. This peptide was isolated and digested by trypsin or *Staphylococcus aureus* V8 protease. Digestion by trypsin yielded four peptides, one of which spanned residues Ala-338–Arg-356 and contained all the bound radioactivity. When trypsin was replaced by V8 protease, a single peptide spanning residues Leu-342–Met-358 was labeled. Edman degradation of the two labeled peptides showed that radioactivity was localized on the following four amino acids: Leu-342, Ile-344, Tyr-345, and Pro-346.

Photolabels derived from ADP and ATP have greatly benefited structural studies of F_1-ATPases. As a preliminary approach, they have been used to locate nucleotide binding sites in F_1 subunits: essentially two types of subunits, α and β, out of the five present in F_1 have been shown to be photolabeled. Photolabeling of F_1 is currently used to map the nucleotide binding sites. Three types of photoactivable derivatives of ADP and ATP have been used [for review, see Vignais & Lunardi (1985)]. They correspond to molecules where the photoreactive group is placed in the 2- or 8-position of the adenine ring (2- and 8-azido nucleotides) or linked to the 3′-carbon atom of the ribose moiety of the nucleotide (3′-arylazido nucleotides and 3′-benzophenone nucleotides). The localization of an azido group on the adenine ring may be advantageous for its proximity to amino acid residues at the active site of the enzyme. 2-Azido-ADP binds to mitochondrial F_1 with a high affinity (K_d = 5 μM) similar to the affinity of ADP (K_d = 2–4 μM) (Boulay et al., 1985). On the other hand, 8-azido-ATP reacts with F_1 with a lower affinity (K_M = 0.5 mM) than ATP (K_M = 0.12 mM) (Wagenvoord et al., 1979). This is probably due to the fact that 2-azido-ADP adopts preferentially an "anti" conformation (Czarnecki, 1984), as do the natural nucleotides (Davies & Danyluck, 1974). In contrast, the "syn" conformation is preferentially taken up by nucleotides with a bulky group at C8 of the purine ring (Sarma et al., 1974). Another striking difference between 2-azido and 8-azido nucleotides is that the former type of probe binds essentially to the β subunit of mitochondrial F_1, whereas the second type is able to label both the α and β subunits (Wagenvoord et al., 1979, 1980). A mapping study of the binding site for 8-azido-ATP in the β subunit of the beef heart mitochondrial F_1 has been recently reported (Hollemans et al., 1983). 8-Azido-ATP was found to bind to two regions. One region corresponded to residues 1–12; in the other region, the photolabel was localized predominantly on three amino acids corresponding to positions 301, 304, and 311. In the present study, we have photolabeled beef heart mitochondrial F_1 with 2-azido[α-^{32}P]ADP. The photolabeled β subunit was cleaved by cyanogen bromide, and the labeled peptide fragment was further digested either by trypsin or by *Staphylococcus aureus* V8 protease. Direct sequencing of the resulting short labeled peptides indicated that the photolabel was attached at positions 342 and 344–346 of the β subunit. Differences in the sites of labeling of the β subunit by the 8-azido and 2-azido nucleotides are discussed.

Experimental Procedures

Materials. $H_3{}^{32}PO_4$ (10 mCi) was purchased from New England Nuclear. All the chemical reagents were of the highest purity available; those utilized to synthesize 2-azido-[α-^{32}P]ADP were redistilled. 2-Azido-[α-^{32}P]ADP was synthesized as described by Boulay et al. (1985), with a specific activity ranging between 800 and 1000 dpm/pmol, depending on the preparation. Beef heart mitochondria were prepared as described by Smith (1967). Beef heart mitochondrial F_1 was prepared according to the method of Knowles et al. (1972) modified by Klein et al. (1982) and was stored at 4 °C, as an ammonium sulfate precipitate. Before use, the suspension was centrifuged, and the pellet was solubilized in a buffer consisting of 50 mM (Tris)[1] base, 50 mM MES, and 2 mM $MgCl_2$, final pH 7.4 (TMM buffer), and desalted by chromatography on an ACA 202 column (IBF) equilibrated with TMM buffer. The ATPase activity of the purified enzyme was tested with a regenerating medium (Pullman et al., 1960); it ranged between 85 and 95 μmol of ATP hydrolyzed·mg^{-1}·min^{-1} at 30 °C.

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* Address correspondence to this author at DRF/Biochimie, C.E.N.-G. 85X, 38041 Grenoble Cedex, France.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid: CNBr, cyanogen bromide; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene 4′-thiohydantoin; DITC, p-phenylene diisothiocyanate; PITC, phenyl isothiocyanate; NaDodSO$_4$, sodium dodecyl sulfate; TFA, trifluoroacetic acid; DTT, dithiothreitol; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Nbf, nitrobenzofurazan; DCCD, dicyclohexylcarbodiimide.

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Photoaffinity Labeling of F_1 with 2-Azido-[α-^{32}P]ADP and Recovery of the Photolabeled β Subunit. Desalted F_1 contained 3.7 ± 0.2 mol of nucleotide/mol of F_1. It was first incubated at a concentration of 10 μM in TMM buffer for 30 min in darkness with 40 μM 2-azido-[α-^{32}P]ADP. The preincubation in the dark was followed by irradiation for 25 s with a Xenon XB100 lamp (1000 W) equipped with a parabolic reflector. As shown by Boulay et al. (1985), short periods of incubation of mitochondrial F_1 with 2-azido-[α-^{32}P]ADP in the dark led to the rapid binding of 3 mol of label/mol of F_1, probably by exchange with readily exchangeable bound ADP or ATP at the level of β subunits or binding to empty nucleotide binding sites. As mitochondrial F_1 contains 3 mol of tightly bound ADP plus ATP per mole of enzyme (Cross & Nalin, 1982), it is concluded that, out of the 3.7 mol of ADP plus ATP present in our F_1 preparation, 0.7 mol of nucleotides was exchangeable with the added azido-[α-^{32}P]ADP and that more label could bind to available empty nucleotide binding sites. To remove the unreacted photoreactive probe, 10 mM ADP was added to the photoirradiated F_1 solution. The photolabeled F_1 was recovered as an ammonium sulfate precipitate. Residual traces of unbound 2-azido-[α-^{32}P]ADP were eliminated by dialysis, during dissociation of F_1 into subunits. The dissociation medium consisted of 50 mM sodium succinate, 1 M NaCl, 0.25 M $NaNO_3$, 0.1 mM DTT, and 4 mM EDTA (Issartel et al., 1983). The β subunit was separated from the $\alpha\gamma\delta\epsilon$ complex by chromatography on DE-52 cellulose (Whatman) with a continuous LiCl gradient. The purity of the β subunit was controlled by electrophoresis on 12% polyacrylamide gel in the presence of $NaDodSO_4$ (Laemmli, 1970).

Succinylation of the β Subunit. Lyophilized β subunit was solubilized at a concentration of 5 mg·mL^{-1} in 7 M guanidinium chloride solution (pH 9.0). Succinylation of the amino groups was performed as described by Walker et al. (1980). The succinylated β subunit was then desalted on a column of ACA 202 gel equilibrated with 50 mM ammonium bicarbonate. The fractions containing the succinylated β subunit were pooled and lyophilized. The faster migration of the succinylated β subunit compared to native β in $NaDodSO_4$–polyacrylamide gel electrophoresis provided an easy control assay to assess the extent of the succinylation reaction. Under our experimental conditions, the β subunit was succinylated to virtually 100%.

Peptide Fragmentation and Purification of Peptide Fragments. The succinylated β subunit was cleaved at methionyl residues by treatment with a 10-fold excess of CNBr in 80% formic acid under nitrogen for 5 h at 37 °C. Subfragmentation was achieved by digestion with trypsin (1:50 w/w) in 100 mM ammonium bicarbonate for 6 h at 37 °C or by *S. aureus* V8 protease (1:50 w/w) in the same medium for 15 h at 37 °C. The resulting peptides were fractionated by gel filtration at 4 °C, as detailed in the legends of the figures, or by reverse-phase high-performance liquid chromatography (Waters) using a C_{18}-μBondapack column. In the latter case, the eluates were monitored at 215 nm with a SF 770 Schoeffel spectrophotometer; the injection buffer was 0.5 M ammonium bicarbonate; the elution buffer and the gradient profile used are specified in the legend of Figure 3.

Peptide Nomenclature. For the sake of clarity we have adopted the same peptide nomenclature as that adopted by Runswick et al. (1983). However, three new peptides CB'9, CB9 V8^1, and CB9 V8^2 have been obtained in our study and are reported in the present paper. The CB'9 peptide which spans residues Pro-320–Met-358 of the β subunit was an acidolytic fragment of CB9 (see Figure 4). Peptides CB9 V8^1 (Arg-295–Glu-341) and CB9 V8^2 (Leu-342–Met-358) were generated by cleavage of CB9 with *S. aureus* V8 protease (see Figure 4).

Amino Acid Analysis. Peptides were hydrolyzed under vacuum in a TFA–HCl mixture (1:2 v/v) containing 0.01% phenol at 166 °C for 25 or 50 min as described by Tsugita et al. (1982). Amino acid analysis was performed by HPLC with a CAT EX resin column (Waters) following the instructions of the manufacturers. The amino acid residues were postderivatized with *o*-phthalaldehyde (OPA) and detected by fluorometry at 420 nm.

Sequence Analysis. Manual liquid-phase DABITC/PITC degradation and DABTH amino acid identification were performed as described by Chang et al. (1978) and Chang (1983).

For solid-phase degradation, peptides CB9 V8^2 and CB'9 were immobilized by their C-terminal homoserine residue by using the homoserine lactone method described by Horn and Larsen (1973) except that resin was replaced by CPG-75 glass beads (Serva, Heidelberg) aminated by 3-aminopropyl (2-aminomethyl) groups (Robinson et al., 1971). The yield of coupling was about 50%. For coupling peptide R20 to DITC-activated amino glass beads, the following treatment was applied. First, the N-terminal residue of peptide R20 was reacted with PITC to avoid binding of the terminal NH_2 on the glass beads. Second, the C-terminal arginine (β-Arg-356) was converted by hydrazinolysis to ornithine (Morris et al., 1973). Free hydrazine was removed by passage on a P2 Bio-Gel column (Bio-Rad) equilibrated with 100 mM ammonium bicarbonate. The modified peptide recovered in the eluate was lyophilized and attached to DITC beads as described by Chang (1979). The beads were washed with 0.5 M $NaHCO_3$, water, methanol, and pyridine sequentially. The yield of binding was about 65%. The immobilized peptides were subjected to stepwise degradation by using the DABITC/PITC double coupling method. The DABTH-amino acids were identified by chromatography on small polyamide sheets (Chang, 1978). Between 20 and 30 nmol of peptides was analyzed in a given assay.

Radioactivity was measured by liquid scintillation (Patterson & Greene, 1965). The protein concentration was estimated as described by Bradford (1976). ATP and ADP extracted from F_1 by heating (Issartel et al., 1986) were assayed by a luminescence test (Lundin et al., 1976).

RESULTS

Identification of the Photolabeled Peptide Fragments Resulting from Cleavage of the F_1–β Subunit Photolabeled with 2-Azido-[α-^{32}P]ADP. After photolabeling of mitochondrial F_1 with 2-azido-[α-^{32}P]ADP, the subunits of the photolabeled enzyme were dissociated and then separated by chromatography on DEAE-cellulose. As shown in Figure 1, 88% of the bound radioactivity was localized in the β subunit of F_1 (peak C). The remaining radioactivity was attributed to the $\alpha,\gamma,\delta,\epsilon$ complex (peak A) and the nondissociated F_1 (peak B). At this stage of the purification, it could be calculated that 25% of the β subunits were covalently labeled by 2-azido-[α-^{32}P]ADP. As shown in the elution profile illustrated in Figure 1, the β peak detected by UV absorption preceded the peak of radioactivity. The relative retention of the photolabeled β subunit on DE-52 cellulose by comparison to the unlabeled β subunit is readily explained by the excess of negative charges brought about by bound 2-azido-[α-^{32}P]ADP. Material in peak C amounted to 350 nmol of labeled and unlabeled β subunits. The yield of recovery of β subunits from the 200 nmol of

Table I: Amino Acid Composition of Peptides Resulting from Cleavage of the β Subunit with CNBr, Trypsin, and *S. aureus* V8 Protease[a]

amino acid residue	peptide 1	CB9	peptide 2	CB'9	peptide 3	R18	peptide 4	R20	peptide 5	CB9 V8[1]	peptide 6	CB9 V8[2]
Asp	5.8	6	3.2	3	4.3	4	2.0	2	4.1	4	2.1	2
Thr	8.6	9	4.0	4	8.6	8	1.0	1	8.1	8	1.3	1
Ser	4.0	4	2.7	3	3.0	3	2.0	2	3.2	3	2.0	2
Glu	4.2	4	1.9	2	1.2	1	1.4	1	1.4	1	0.8	0
Gly	2.3	2	2.0	1	1.3	1	1.6	1	1.2	1	1.3	1
Ala	8.8	9	5.6	7	6.2	6	3.3	3	7.5	8	1.3	1
Val	4.5	5	2.0	2	3.2	4	0.9	1	3.0	4	0.9	1
Met	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
Ile	4.8	6	2.3	3	2.7	3	1.8	2	3.6	4	1.6	2
Leu	4.8	5	3.7	4	3.1	3	2.3	2	3.2	3	2.0	2
Tyr	1.6	2	0.5	1	0.5	1	0.4	1	0.9	1	0.5	1
Phe	1.0	1	1.0	1	0.9	1	0.1	0	1.1	1	0.0	0
His	1.2	1	0.9	1	0.9	1	0.2	0	0.5	1	0.1	0
Lys	1.4	2	0.0	0	1.6	2	0.2	0	2.0	2	0.1	0
Arg	2.7	3	1.6	2	1.0	1	0.9	1	0.9	1	0.9	1

[a] Peptides 1–6 were obtained as described under Results and the corresponding figures (peptides 1 and 2, Figure 3; peptides 3 and 4, Figure 5; peptides 5 and 6, Figure 6). Their amino acid compositions are compared to those of peptides CB9, CB'9, R18, R20, CB9 V8[1], R18, and CB9 V8[2] (Runswick & Walker, 1983; Results).

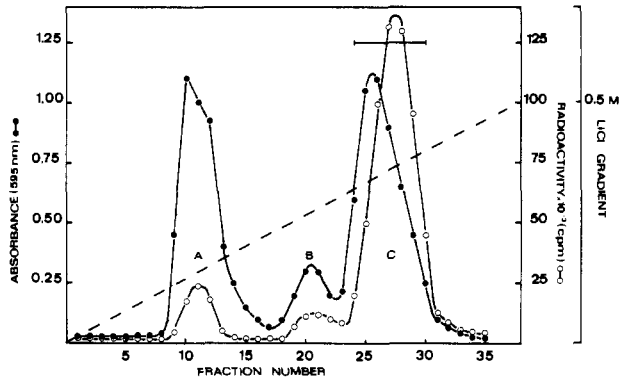

FIGURE 1: Fractionation of subunits of beef heart F_1 photolabeled with 2-azido-[α-^{32}P]ADP. Two hundred nanomoles of photolabeled F_1 in TMM buffer was submitted to photolabeling, dialysis, and dissociation as described under Experimental Procedures. The dissociated F_1 was loaded onto a column (30 × 1.6 cm) of DE-52 cellulose (Whatman) equilibrated with 25 mM succinate, 5 mM ATP, 4 mM EDTA, and 2.5 mM β-mercaptoethanol. Elution was performed with a linear 0–0.5 M gradient of LiCl. Three-milliliter fractions were collected. Twenty-microliter samples were taken from each fraction for determination of radioactivity and protein concentration. Fractions 24– to 30 were pooled (indicated by the bar).

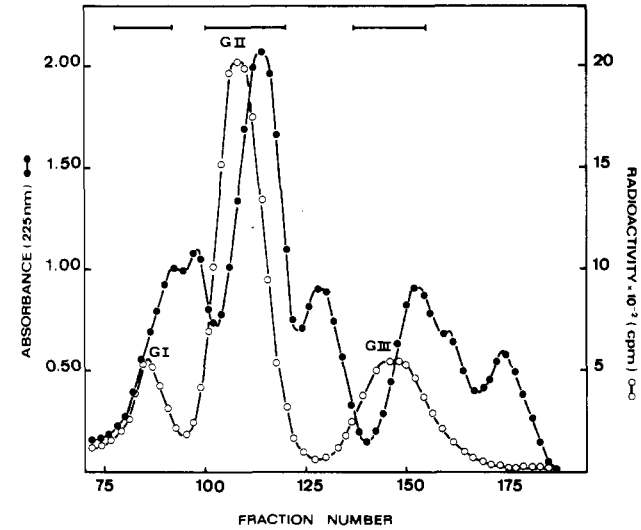

FIGURE 2: Fractionation of CNBr cleavage products of photolabeled β subunit. The CNBr cleavage fragments (see Experimental Procedures) were fractionated by chromatography on a Sephadex G-75 superfine column (120 × 2 cm) in 50 mM ammonium bicarbonate. Two-milliliter fractions were collected. The bars correspond to the pooled fractions. The recovery of the radioactive material in the eluates was 87%.

photolabeled F_1 was close to 60% on the basis of a stoichiometry of three β subunits per F_1.

After succinylation, the β subunit was cleaved by CNBr, and the cleavage products were fractionated by filtration on Sephadex G-75. The UV profile was similar to that reported by Runswick and Walker (1983). The radioactivity profile consisted of three peaks GI, GII, and GIII (Figure 2) containing 11%, 60%, and 29% of the bound radioactivity, respectively.

The material of the more radioactive peak, GII, was subjected to HPLC,using a gradient of acetonitrile (Figure 3). By this technique, it was possible to recover a strongly radioactive peak with exact superimposition of absorbance and radioactivity. The more radioactive fractions 13–16 were pooled; the peptide material (peptide 1) had the same amino acid composition (Table I) and the same N-terminal amino acid, namely, glutamine, as the peptide fragment CB9 [nomenclature of Runswick & Walker (1983)] spanning residues Gln-293–Met-358. The less radioactive peptide material contained in fractions 17–24 (Figure 3) was freeze-dried, solubilized in a small volume of 0.5 M ammonium bicarbonate, and reinjected in the HPLC column; essentially one peak was obtained with a similar retention time as that observed for fractions 13–16 in the first fractionation. The first peak (fractions 4–7, Figure 3) corresponded to the void volume; as its amino acid composition was similar to that of fractions 13–16, it could be concluded that it consisted of aggregates of peptide CB9.

The material of peak GIII (Figure 2) was fractionated by HPLC with the same acetonitrile gradient as that used for fractionation of the material of peak GII. Likewise, one strongly radioactive peak was obtained (peptide 2, Table I), whose N-terminal sequence (PAPAVTFAH) and amino acid composition (Table I) corresponded to the peptide spanning Pro-320–Met-358. Because this peptide is a portion of CB9, it will be referred to as CB'9 (Figure 4). As shown by Landon (1977), the aspartyl–prolyl bond is acido labile; most likely CB'9 was derived from CB9 by acidolytic cleavage at the Asp-319–Pro-320 bond during CNBr attack in 80% formic acid.

The partial cleavage products of subunit β contained in peak GI were digested by trypsin, and the digest was chromato-

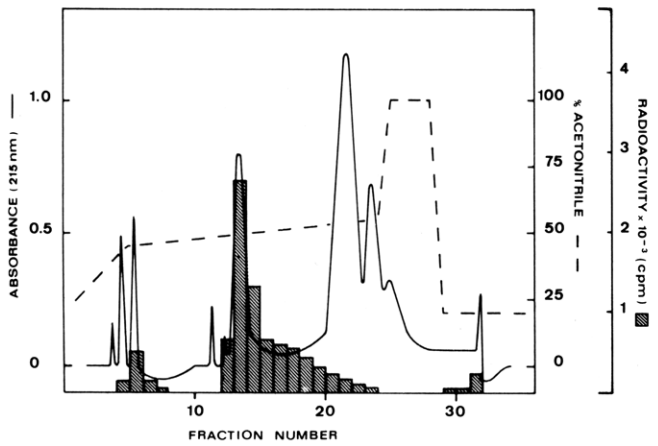

FIGURE 3: Purification of the major radioactive peptide obtained by CNBr cleavage by HPLC. The column of C18 μBondapack was equilibrated in 80% buffer A (0.1% TFA) and 20% buffer B (0.1% TFA, 99.9% acetonitrile). The material corresponding to the GII fraction (Figure 2) was injected, and the column was subjected to an acetonitrile gradient at a flow rate of 1 mL min^{-1}. Ten-microliter fractions were collected at 1-min intervals and analyzed for the radioactivity content. A total of 82% of the radioactivity present initially in the chromatographed sample was recovered by elution; 60% of the recovered radioactivity was in fractions 13–16, 28% in fractions 17–35, and 12% in fractions 4–7.

graphed on a P4 Bio-Gel column (120 × 1.5 cm) equilibrated in 100 mM ammonium bicarbonate. A main radioactive peak was obtained, whose amino acid composition and N-terminal sequence were the same as those of peptide R20, spanning Ala-338–Arg-356 (Figure 4).

At this stage of the fractionation of the photolabeled β subunit, it could be calculated that more than 95% of the bound radioactivity was located in peptide CB9 or its cleavage products, namely, peptides CB′9 and R20.

Exploration of Bound Photolabel in the Peptide Fragment of Photolabeled CB9 after Digestion by Trypsin and S. aureus V8 Protease. A 50-nmol sample of photolabeled CB9 obtained as described in the preceding section was treated by trypsin (cf. Experimental Procedures), and the tryptic digest was fractionated by Bio-Gel chromatography (Figure 5). The elution pattern showed a main peak of absorbancy corresponding to fractions 63–69 (peak I) and a minor peak with a shoulder (fractions 73–82) (peak II) which contained 97% of the recovered radioactivity. On the basis of the amino acid composition (Table I) and the N-terminal amino acid, it was concluded that the two peptides contained in peaks I and II (Figure 5) referred to as peptides 3 and 4 in Table I were R18 and R20, respectively (cf. Figure 4 and Table I). All the fractions of peak II contained pure peptide R20; the shoulder in the elution pattern was therefore explained by differential elution of labeled and unlabeled fragments of peptide R20. As a matter of fact, 2-azido-ADP (M_r 468) brings a significant increment to the molecular weight of the peptide to which it is attached. A similar differential elution was observed in the Sephadex G-75 chromatography illustrated in Figure 2.

The other enzymatic digestion was carried out with *S. aureus* V8 protease. Thirty nanomoles of photolabeled CB9 was digested by V8 protease. The UV absorption and radioactivity profiles are given in Figure 6. The first peak (fractions 53–57) contained about 10% of the recovered radioactivity. The material of this peak is referred to as peptide 5 in Table I, and it corresponds to peptide CB9 $V8^1$ (Figure 4). A second digestion of this peptide by the V8 protease did not modify the pattern of elution of radioactivity. The remaining radioactivity (90%) was eluted in peak II (fractions 62–72) corresponding to peptide 6 (Table I) which is identical with peptide CB9 $V8^2$. As for elution of the tryptic digest, the shoulder of peak 2 (fractions 62–67) could be explained by bound azido-ADP.

In summary, from the cleavage experiments carried out with trypsin and V8 protease, it is clear that bound 2-azido-[α-^{32}P]ADP is localized in two overlapping fragments, R20 (Ala-338–Arg-356) and CB9 $V8^2$ (Leu-342–Met-358). The peptide CB9 $V8^1$ obtained together with CB9 $V8^2$ contains 10% only of the photolabel bound to CB9. As CB9 $V8^1$ represented a minor radioactive fraction, it was not further investigated. It is possible, but not proven, that this small amount of bound radioactivity is attached to the portion of R20 which overlaps CB9 $V8^1$, namely, Ala-338–Glu-341.

Sites of Covalent Attachment of 2-Azido-[α-^{32}P]ADP. The two radioactive peptides R20 and CB9 $V8^2$ were attached to glass beads and sequenced as described under Experimental Procedures. The radioactivity profiles (Figure 7) corresponding to the two Edman degradations were in perfect agreement in showing that in both peptides 2-azido-[α-^{32}P]-ADP is attached to Leu-342, Ile-344, Tyr-345, and Pro-346.

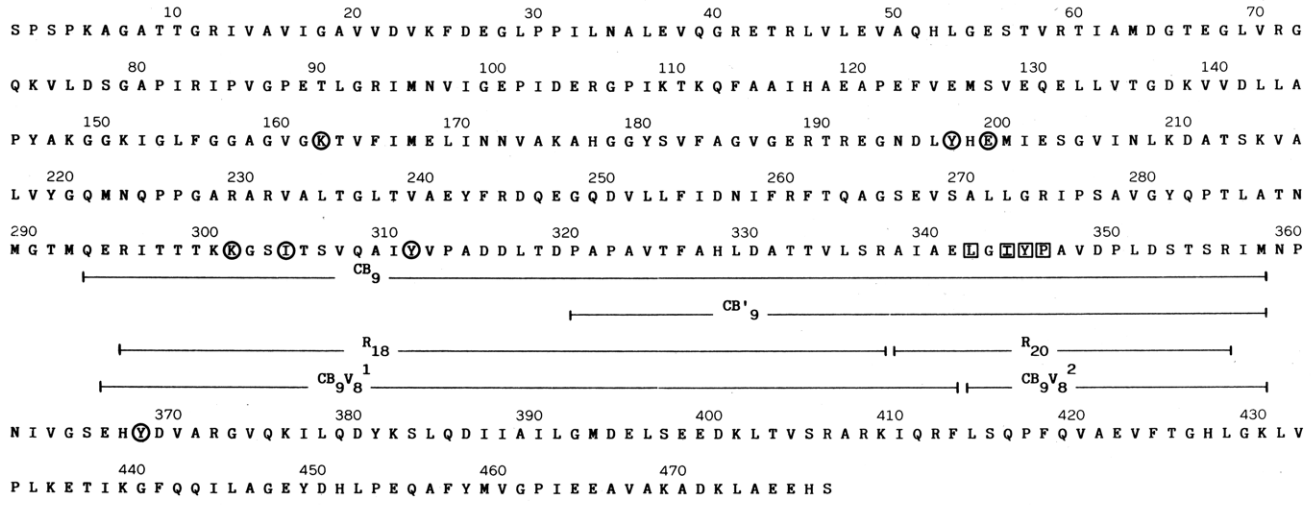

FIGURE 4: Localization of radioactivity in the amino acid sequence of the β subunit of bovine heart mitochondrial F_1-ATPase after photolabeling with 2-azido-[α-^{32}P]ADP. Radioactivity was localized in peptides CB9, CB′9, R20, and CB9 $V8^2$ (see text), precisely on residues 342, 344, 345, and 346 (surrounded by a square). Circled residues are the sites of labeling with dicyclohexylcarbodiimide and 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (residue 199), 7-chloro-4-nitro-[^{14}C]benzofuran (residues 162, 197, and 311), 8-azido-[2-^{3}H]ATP (residues 301, 304, and 311) and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (residue 368).

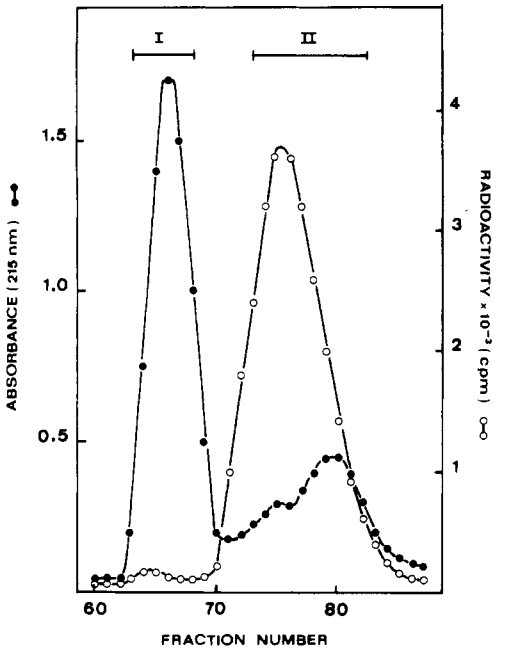

FIGURE 5: Fractionation of a trypsin digest of photolabeled CB9 by gel chromatography. Fifty nanomoles of photolabeled CB9 in 100 mM ammonium bicarbonate was digested by trypsin as described under Experimental Procedures. The digest was chromatographed on a P6 Bio-Gel column (1.5 × 100 cm) equilibrated with 100 mM ammonium bicarbonate. One-milliliter fractions were collected from which 20 μL was withdrawn for determination of radioactivity. A total of 96% of the loaded radioactivity was recovered.

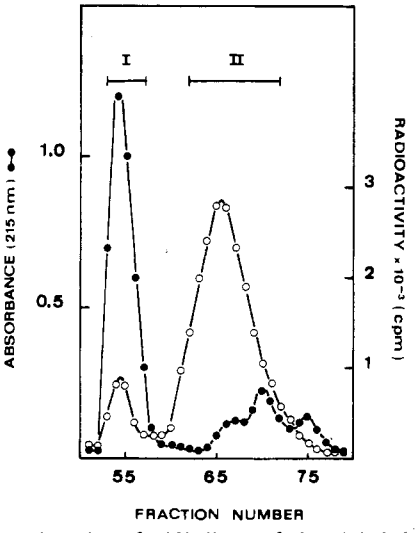

FIGURE 6: Fractionation of a V8 digest of photolabeled CB9 by gel chromatography. Thirty nanomoles of labeled CB9 in 100 mM ammonium bicarbonate was digested by *S. aureus* V8 protease. The digest was chromatographed on a Bio-Gel P4 column (1.5 × 100 cm) equilibrated with 100 mM ammonium bicarbonate. One-milliliter fractions were collected from which 20-μL samples were withdrawn for radioactivity determination. A total of 93% of the loaded radioactivity was recovered.

The Tyr residue was more labeled than the others. One cannot exclude that the labeling attributed to Pro-346 may be the result of an incomplete Edman Degradation at the level of Tyr-345 and that the radioactivity recovered at Pro-346 may represent carryover.

Sometimes, in the first cycle of degradation of an attached peptide, that part of the peptide which is not firmly attached to the beads is eluted with the first amino acid residue. This may be prevented by extensive washing with TFA prior to the first cycle of degradation. TFA washing was indeed applied

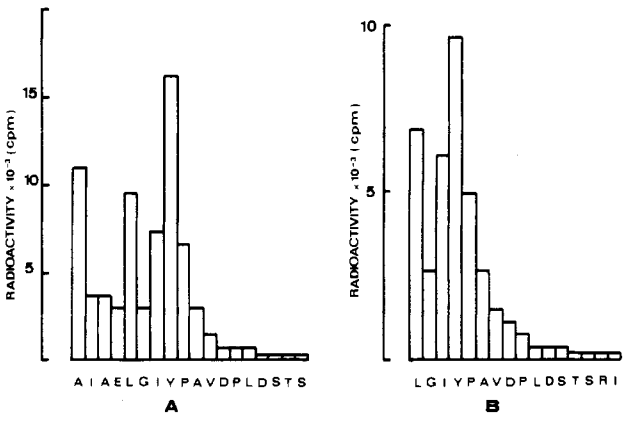

FIGURE 7: Edman degradation of the radiolabeled peptides R20 (A) and CB9 $V8^2$ (B) and release of radioactive amino acid residues. The immobilized peptides were sequenced as described under Experimental Procedures.

to CB9 $V8^2$, but not to R20 which had its terminal NH_2 blocked with PITC (see Experimental Procedures). Consequently, no firm conclusion could be drawn about the labeling of the N-terminal alanine in R20; however, as previously mentioned, the radioactivity bound at this level of R20 represented no more than 10% of the radioactivity contained in the entire sequence of R20.

Discussion

Methodological Aspects. To ascertain the specific binding of the 2-azido-ADP to the β subunit of mitochondrial F_1, the molar ratio of the added label to F_1 was fixed at a value of about 4. Under these conditions, only a fraction (25%) of the β subunits was labeled, but the reaction of the photoprobe with the nucleotide binding site of the β subunit was ensured. It was checked that the conditions of photoirradiation were not harmful to the protein material; in fact, after the 25-s period of photoirradiation which was routinely used, the F_1-ATPase activity (in the absence of 2-azido-[α-^{32}P]ADP) was still 90–94% of the initial value.

For a precise study of the localization of the photoprobe on peptide CB9 obtained from the β subunit by CNBr cleavage, two types of enzymatic digestion of labeled CB9 were undertaken, one with trypsin and the other with the *S. aureus* V8 protease. The resulting radioactive peptides R20 and CB9 $V8^2$ had an overlapping sequence. By Edman degradation, the photoprobe was found to be localized on the same amino acid residues in the two peptides.

It has been reported that photoprobes may interfere with the Edman degradation; a typical case is that of the Rec A protein where photolabeling with 8-azido-ADP prevented amino acid sequencing (Knight & McEntee, 1985). In our study concerning the labeling of mitochondrial F_1 by 2-azido-ADP, the sequencing of the labeled fragment was not prevented.

The 2-azido-ADP used in this work was labeled in the α-P group. Although this labeling required tedious chemical synthesis, we preferred it to the labeling in the β-P group of ADP (Czarnecki, 1982; MacFarlane et al., 1982). In fact, in the course of the cleavage and fractionation steps following photolabeling, labeled F_1 molecule may encounter drastic conditions which result in dephosphorylation of the β-P group of the attached 2-azido-ADP. The labeling of 2-azido-ADP with an α-^{32}P group provided the probe with a more stable radioactivity.

Comparative Localization of 2-Azido-[α-^{32}P]ADP and Other Probes Specific to the β Subunit of Mitochondrial F_1.

In the present work, a discrete sequence (Leu-342–Pro-346) of the β subunit of mitochondrial F_1 was shown to be photolabeled by 2-azido-[α-^{32}P]ADP. The labeled sequence is expected to interact with the purine ring of the nucleotide. There is a strong homology between this sequence and the corresponding region in the β subunits of *Escherichia coli* F_1 and maize and spinach chloroplast F_1 (Walker et al., 1984) and the β subunits of human F_1 (Ohta & Kagawa, 1986). The high degree of conservation of this sequence is probably linked to a strategic function, possibly a role as a nucleotide binding site. The Leu-342–Pro-346 sequence of the β subunit labeled by 2-azido-ADP has not yet been reported to be labeled by other probes. It should be mentioned, however, that in addition to a major site of attachment on Glu-199, the carboxyl reagent EEDQ also binds to Glu-341 (Laikind et al., 1985). The binding site for 2-azido-ADP is close to, but does not contain, the Tyr-368 to which the nucleotide analogue FSBA has been reported to be attached (Esch & Allison, 1978). It should be remembered that 8-azido-ATP binds to the sequence Lys-301–Tyr-311 (Hollemans et al., 1983).

The 2-azido-ADP binding site is quite remote from the binding sites of chemical modifiers that inactivate mitochondrial F_1. For example, it has been found that at neutral pH Nbf binds to Tyr-197 (Ho & Wang, 1983) close to the essential Glu-199 modified by DCCD (Esch et al., 1981) and EEDQ (Laikind et al., 1985). However, in a more recent study, Nbf was reported to bind to Tyr-311 (Andrews et al., 1984a; Sutton & Ferguson, 1985) in the same region as that labeled by 8-azido-ATP. The fact that Nbf and 8-azido-ATP (Hollemans et al., 1983) bind to the same Tyr residue (Tyr-311) might be taken as evidence for the Nbf-dependent inactivation of F_1 by direct binding of the modifier to the nucleotide site. This explanation would be consistent with the finding that binding of Nbf to F_1 prevents photolabeling by 3′-arylazido nucleotides (Lunardi & Vignais, 1979). On the other hand, it is difficult to reconcile with the absence of effect of Nbf on the binding of [^{14}C]ADP to F_1 (Di Pietro et al., 1980). Along this line, it has been suggested that β Tyr-311 is located not at the catalytic site but at a site which participates in conformational changes in response to nucleotide binding (Ferguson et al., 1975; Andrews et al., 1984a). Upon incubation at alkaline pH, Nbf is transferred from a tyrosine to a lysine residue in the β subunit. The modified lysine residue was identified as Lys-162 (Andrews et al., 1984b), apparently far from the amino acid residues labeled by the azido nucleotides. One may, however, conceive that, because of a folded structure, the part of the chain containing Lys-162 is close to the site to which the purine moiety of the azido nucleotides binds. The model proposed thereafter for the nucleotide binding site of the β subunit takes this possibility into account.

Several explanations can be given for the different attachments of 2-azido-ADP (this work) and 8-azido-ATP (Hollemans et al., 1983) to the β subunit of mitochondrial F_1. At first we considered the possibility that the different localization to covalently bound 2-azido and 8-azido nucleotides in the amino acid sequence of F_1 could arise from the difference in length of the phosphate chain of the two probes. F_1 was irradiated in the presence of 2-azido-[α-^{32}P]ATP under similar conditions as described by Hollemans et al. (1983); we found the same distribution of bound radioactivity after photolabeling with 2-azido-[α-^{32}P]ATP and 2-azido-[α-^{32}P]ADP in the tryptic and SA V8 peptides. This does not mean that binding of ATP and ADP to F_1 does not result in different conformations of the enzyme, a possibility which was actually discussed in a recent paper (Vignais et al., 1984). Should this change of conformation occur, the results presented here would suggest that it does not concern the site of attachment of the purine ring to the nucleotide binding site of F_1 but probably that of the phosphate chain of the nucleotide.

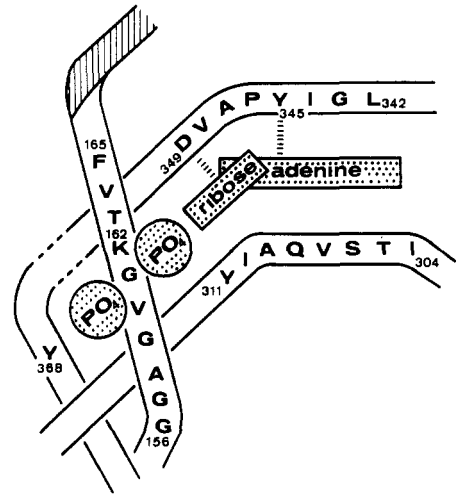

FIGURE 8: Diagram representation of possible interaction of ADP with the amino acid residues belonging to the nucleotide binding site of the β subunit.

The second explanation for the different attachments of 2-azido-ADP and 8-azido-ATP is based on the goodness of fit of the two probes to the nucleotide binding site of the β subunit of F_1. For example, one may assume that the nucleotide binding site of the β subunit accommodates 2-azido-ADP and 8-azido-ATP in spite of their different conformations. 2-Azido-ADP is believed to have a preferential "anti" conformation (Czarnecki, 1984) typical of natural nucleotides (Davies & Danyluck, 1974), whereas the presence of the azido group at the C8 position of the purine favors the "syn" conformation (Sarma et al., 1974). The fact that the 2-azido-ADP, but not the 8-azido-ATP, mimics natural ADP is reflected by the higher affinity of 2-azido-ADP compared to 8-azido-ATP. In other words, 8-azido-ATP would not adapt easily to the geometry of the nucleotide binding site because of its syn conformation in contrast to 2-azido-ADP. Along this line, we should like to favor the idea that the low affinity of 8-azido-ATP for F_1 is due to the minor fraction of the reactive anti conformation in equilibrium with the syn conformation in 8-azido-ATP. The "reactive" 8-azido-ATP and 2-azido-ADP would bind to the same nucleotide binding center. The differences in the amino acid residues reacting with 8-azido-ATP and 2-azido-ADP is explained as follows. On the basis of the requirement of a folded structure for nucleotide binding sites (Rao & Rossmann, 1973), it is possible that the Lys-301–Tyr-311 sequence (Hollemans et al., 1983) and the Leu-342–Pro-346 sequence (this paper) constitute the walls of a crevice in the β subunit, in which the bound 8- and 2-azido nucleotides are located. The distance between C2 and C8 in the purine ring is close to 6 Å; 2- and 8-azido groups are therefore expected to react with different amino acid residues of the crevice. In keeping with this hypothesis and by analogy with the structures of the nucleotide binding sites in proteins P21 (Mc Cormick et al., 1985) and EF-Tu (Jurnak, 1985), we propose in Figure 8 a model for the interaction of nucleotides with their binding site in the β subunit of mitochondrial F_1. In previous models presented by Cross (1981) and Wang (1983), a Tyr residue was placed near the adenine ring of the bound nucleotide. On the basis of our experimental data, β Tyr-345 might be this residue; it could interact by

hydrogen bonding with a N atom of the adenine ring. The Asp-349 residue which is close to the reactive sequence might establish a H bond with the 2'-OH group of the ribose moiety of ADP, as is the case for a number of dinucleotide binding proteins (Wierenga et al., 1985). The sequence G-X-X-X-X-G-K is common to many enzymes that bind mono- and dinucleotides (Möller & Amons, 1985); it is also found in the β subunit of mitochondrial F_1 (Gly-156–Lys-162). The glycine residues of this sequence allow a high degree of flexibility and could be responsible for a close contact between the sequence and the phosphate chain of ADP or ATP, as illustrated in Figure 8. The folded structure shown in Figure 8 would be consistent with the transfer of bound Nbf from Tyr-311 to Lys-162 at alkaline pH (as mentioned earlier).

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Registry No. ATPase, 9000-83-3; ADP, 58-64-0; ATP, 56-65-5; L-Leu, 61-90-5; L-Ile, 73-32-5; L-Tyr, 60-18-4; L-Pro, 147-85-3; 2-azidoadenosine diphosphate, 64020-53-7.

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