Photoaffinity Labeling of Mitochondrial Adenosine Triphosphatase by an Azido Derivative of the Natural Adenosine Triphosphatase Inhibitor†

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ABSTRACT: The natural mitochondrial ATPase inhibitor (IF_1) was modified with a radioactively labeled heterobifunctional and photosensitive reagent, methyl 4-azido(^{14}C)benzimidate (^{14}C)MABI). Titration experiments of IF_1 by (^{14}C)MABI and tryptic maps of (^{14}C)MABI-IF_1 indicated that specific lysine residues in IF_1 are preferentially labeled by (^{14}C)MABI. Under appropriate conditions of labeling (1 to 2 lysine residues modified per IF_1), MABI-IF_1 exhibited the same inhibitory potency as native IF_1 on the hydrolytic activity of the coupling factor 1 of mitochondrial ATPase (F_1). The same conditions were required for inhibition of F_1 by MABI-IF_1 and IF_1 (slightly acidic pH and presence of ATP and $MgCl_2$). In photolabeling experiments, (^{14}C)MABI-IF_1 was used to investigate the localization of IF_1 binding sites on F_1. Upon photoirradiation, MABI-IF_1 bound selectively to the β subunit of soluble or membrane-bound F_1. Adenylyl imidodiphosphate and quercetin, two compounds which partially mimic the inhibitory effect of IF_1 on ATPase activity of F_1, markedly prevented the binding of (^{14}C)MABI-IF_1 to F_1; on the other hand, aurovertin, a specific ligand of the β subunit of F_1, did not affect the interaction between (^{14}C)MABI-IF_1 and F_1. In the absence of light, (^{14}C)MABI-IF_1 was used as a reversible radiolabeled ligand with respect to membrane bound F_1 to investigate F_1-IF_1 interactions in inside-out submitochondrial particles as a function of the energy state of the particles. Oxidation of NADH by submitochondrial particles resulted in a decrease of bound (^{14}C)MABI-IF_1; the effect was counteracted by antimycin. The data suggested that added (^{14}C)MABI-IF_1 is capable of exchanging with IF_1 bound to F_1 in submitochondrial particles and that the rate and extent of (^{14}C)MABI-IF_1 release are triggered by the proton-motive force developed by the particles.

The mitochondrial ATPase inhibitor (IF_1)[1] (Pullman & Monroy, 1963) is a heat-stable protein of low molecular weight (less than 10 000) which is loosely bound to the F_1 sector of ATPase [for recent reviews, see Pedersen et al. (1978), Penefsky (1979), and Senior (1979)]. It is recognized that IF_1 inhibits both ATP-dependent reactions in mitochondria (Asami et al., 1970; Ernster et al., 1973; Van de Stadt et al., 1973; Van de Stadt & Van Dam, 1974) and oxidative phosphorylation (Harris et al., 1979). In order to elucidate the mechanism of this inhibition in terms of molecular interactions, radiolabeled IF_1 was required. In the first labeling experiments, IF_1 from yeast mitochondria was biosynthetically labeled by ^{14}C by growing the yeast cells in a medium supplemented with (^{14}C) leucine (Satre et al., 1975). In more recent experiments, IF_1 prepared from beef heart mitochondria was radiochemically labeled by (^{14}C)PITC, resulting in a much higher specific radioactivity (Klein et al., 1980).

In the present work, IF_1 was modified by (^{14}C)MABI, a reagent which possesses an imido ester at one end and a photoactivable azido group at the other end. (^{14}C)MABI was covalently bound in the dark by its imidate group to IF_1 (most likely at the level of lysine residues); then (^{14}C)MABI-IF_1 was added to F_1. Upon photoirradiation, the azido group of (^{14}C)MABI-IF_1 was activated to give a reactive nitrene which is expected to bind covalently to the same subunit of F_1 as that recognized by the IF_1 moiety of (^{14}C)MABI-IF_1. On the basis of this principle, the experiments reported here describe the use of (^{14}C)MABI-IF_1 to localize the binding site of IF_1 on F_1 and to investigate the effect of a number of F_1 ligands on F_1–IF_1 interactions. (^{14}C)MABI-IF_1 was also used, in the absence of light, in reversible binding assays, to analyze the changes in binding affinity of IF_1 for membrane-bound F_1 in submitochondrial particles placed in different metabolic conditions.

Materials and Methods

Cold MABI was obtained from Pierce. (^{14}C)MABI (11 mCi/mmol) was synthesized by the Commissariat à l'Energie Atomique, Saclay, France. (^{14}C)DCCD (54.5 mCi/mmol) and (^{14}C)NEM (23.7 mCi/mmol) were obtained from the Commissariat à l'Energie Atomique, Saclay, France, and from the Radiochemical Centre, Amersham, England respectively.

Biological Preparations. Beef heart mitochondria were prepared as described by Smith (1967). Beef heart submitochondrial particles depleted of their endogenous inhibitor protein (AS particles) were prepared by the method of Racker & Horstman (1967). MgATP particles were prepared according to Löw & Vallin (1963). Coupling factor F_1 was purified by the method of Knowles & Penefsky (1972). The purified F_1 had a specific activity of 70–80 μmol of ATP hydrolyzed min^{-1} mg^{-1} when assayed as described by Knowles & Penefsky (1972). F_1 was stored as a 2 M $(NH_4)_2SO_4$ precipitate in 0.25 M sucrose, 50 mM Tris-HCl, 2 mM EDTA, and 4 mM ATP, pH 8.0. Before use, the F_1 was desalted by passage through a Sephadex G-50 (fine) column as described by Penefsky (1977). ATPase inhibitor was purified by the method of Horstman & Racker (1970) as modified by Kagawa

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[1] Abbreviations used: AMPPNP, adenylyl imidodiphosphate; AS particles, submitochondrial particles prepared from beef heart mitochondria by sonication in the presence of ammonium hydroxide at pH 9.0 followed by a Sephadex G-50 treatment; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; F_1, beef heart mitochondrial coupling factor; IF_1, beef heart ATPase protein inhibitor; $(IF_1)_2$, $(IF_1)_3$, dimer, trimer, etc., of IF_1; MABI, methyl 4-azidobenzimidate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; P_i, inorganic phosphate; PITC, phenyl isothiocyanate; TPCK-trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin; EDAC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

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(1974) for the ethanol fractionation step.

Derivatization of IF_1 by MABI or (^{14}C)MABI. All steps were carried out in the dark. MABI was added to 1 mg of IF_1 (2 mg/mL) in 100 mM triethanolamine buffer, pH 9.0, to a final concentration of 4.7 mM. The mixture was incubated for 1 h at 37 °C with constant stirring. Then 10 mM Tris-SO_4, pH 8.0, was added to inactivate the nonreacted MABI. MABI-IF_1 was separated from free MABI on a Sephadex G-25 (medium) column (1.5 × 20 cm) and eluted in the void volume. (^{14}C)MABI-IF_1 was prepared according to the same procedure. Its specific radioactivity was between 10 and 15.10^9 cpm/mmol.

Covalent Photolabeling of F_1 by MABI-IF_1. Photoactivation of MABI-IF_1 was done with UV light delivered by a mineral light UVS11 lamp placed at a distance of 5 cm from the sample. In routine irradiation MABI-IF_1 (7 μg) and F_1 (75 μg) were preincubated at 25 °C in the dark for 15 min before UV irradiation in 0.25 mL of a medium containing 0.25 M sucrose, 1 mM $MgCl_2$, 0.5 mM ATP, and 10 mM MOPS, final pH 6.5, for 5 min. UV light under the described conditions had no significant effect on the activity of F_1 or IF_1.

Enzyme Test. The assay of residual ATPase activity following binding of IF_1 or MABI-IF_1 to F_1 was performed as described by Horstman & Racker (1970). The incubation was initiated by addition of 0.25 mL of an ATPase assay medium containing 0.1 M Tris-SO_4, 20 mM ATP, 10 mM $MgCl_2$, 20 mM phosphoenolpyruvate, and 30 μg of pyruvate kinase, final pH 8.0. It was carried out for 10 min at 30 °C and terminated by the addition of 0.125 mL of 2.5 M $HClO_4$. Inorganic phosphate (P_i) was determined on 0.2 mL aliquots by the method of Fiske & SubbaRow (1925).

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide gels containing 0.1% $NaDodSO_4$ was carried out as described by Weber & Osborn (1969). After overnight migration at 4 mA/gel, the gels were stained for 4–6 h with a solution made of 0.05% Coomassie Blue R 250, 25% isopropyl alcohol, and 10% acetic acid and destained according to the method of Fairbanks et al. (1971). The densitometric traces were recorded with a Joyce Loebl Scan 400. For determination of the distribution of radioactivity, the gels previously stained and destained were frozen in solid CO_2 and sliced in 1-mm slices with a Joyce Loebl slicer. Slices were digested by overnight incubation in 1 mL of 10% H_2O_2 at 50–60 °C and counted in 10 mL of a scintillation fluid (Patterson & Greene, 1965).

Protein Concentration. The protein concentration of F_1 and IF_1 samples was determined by the method of Bradford (1976) with Coomassie Blue G250 and by the biuret method of Gornall et al. (1949) for submitochondrial particles. Bovine serum albumin was used as a standard.

Tryptic Peptide Mapping. The peptide mapping was performed as described previously (Klein et al., 1980). (^{14}C)-MABI-IF_1 (40 μg of protein, 0.9 mol of (^{14}C)MABI/mol of IF_1) in 30 mM ammonium bicarbonate, pH 7.9, was incubated at 37 °C for 3 h with TPCK-trypsin in a final volume of 0.11 mL, using a trypsin to IF_1 ratio of 1:100, and for another period of 3 h after a new addition of TPCK-trypsin to bring the trypsin to IF_1 ratio to 1:50. Then 0.02 mL of 1 N acetic acid was added and the digest was lyophilized. The lyophilized digest was redissolved in 3 μL of electrophoresis buffer (see below), spotted on a 10 × 10 cm cellulose thin layer plate (F1440, Schleicher and Schüll), and subjected to electrophoresis at 200 V for 1 h at 4 °C in pyridine/acetic acid/acetone/H_2O (20:40:160:800 v/v) pH 4.4. Ascending chromatography in butanol/pyridine/acetic acid/H_2O (30:20:6:24 v/v) was performed in the second dimension for 2 h.

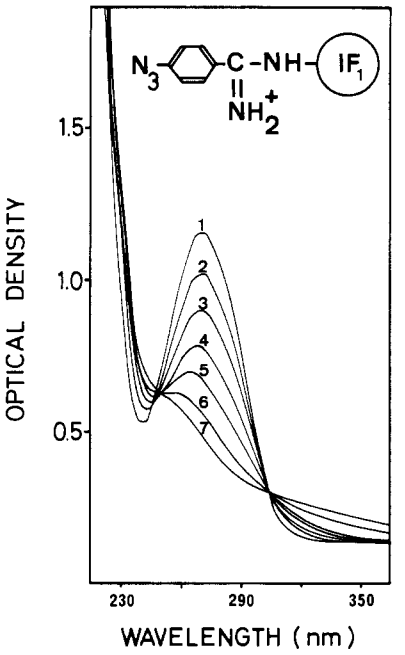

FIGURE 1: Absorption spectra of methyl 4-azidobenzoimidate-IF_1 (MABI-IF_1). A 0.4 mg/mL solution of MABI-IF_1 in 10 mM ammonium sulfate, pH 8.0, was irradiated in a 1-cm optical length quartz cuvette with mineral light UVS 11. The figure shows that the peak of 273 nm was gradually decreased upon irradiation (traces 1–7).

Results

Chemical and Biological Properties of (^{14}C)MABI-IF_1. (^{14}C)MABI-IF_1 prepared in the dark as described under Materials and Methods was freed of contaminating (^{14}C)-MABI by Sephadex chromatography. Under these conditions, MABI is expected to react by its imidate group with lysine residues in IF_1. The extinction coefficient of the absorption peak at 273 nm of (^{14}C)MABI-IF_1 was calculated to be 20 000 on the basis of the (^{14}C)MABI incorporated and the specific radioactivity of (^{14}C)MABI, in good agreement with the MABI extinction coefficient of 17 000 reported by Ji (1977). When MABI-IF_1 was irradiated under UV light, the peak at 273 nm was gradually reduced (Figure 1), in accordance with the photosensitivity of MABI-IF_1.

The inhibitory effect of MABI-IF_1 on F_1-ATPase activity was tested in the dark against AS particles. Labeling of IF_1 by (^{14}C)MABI to various extents was achieved by sequential additions of (^{14}C)MABI to IF_1 in 100 mM triethanolamine, pH 8. As shown in Figure 2, up to 5 MABI residues could be incorporated without any loss of biological activity of IF_1. Parallel titration of ATPase in AS particles by native IF_1 and by IF_1 modified with about 1 mol of MABI per mol of enzyme showed that partially modified MABI-IF_1 behaves exactly like IF_1 (Figure 3).

Tryptic Map of (^{14}C)MABI-IF_1. Under the described conditions, tryptic digestion was complete. A tryptic map of (^{14}C)MABI-IF_1 modified with an average of 1 MABI residue per mol of IF_1 is presented in Figure 4. The tryptic peptides were revealed by ninhydrin staining and autoradiography. Comparison of stained tryptic peptides from unmodified IF_1 and MABI-IF_1 revealed the same spots (about 17). Three highly radioactive peptides were detected, together with several minor ones. The accumulation of a restricted number of radioactive peptides suggested a preferential reactivity of MABI with specific lysine residues in IF_1. The tryptic map of MABI-IF_1 was similar to the tryptic map of IF_1 modified

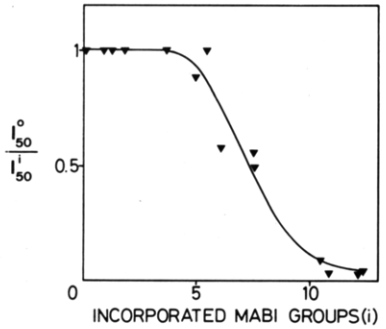

FIGURE 2: Effect of MABI derivatization on IF_1 inhibitory activity. IF_1 was incubated with (^{14}C)MABI in the dark under the conditions described under Materials and Methods. (^{14}C)MABI was incorporated into IF_1 to different extents (up to 12 mol of MABI/mol of IF_1) by stepwise addition of (^{14}C)MABI. The different samples of (^{14}C)-MABI-IF_1 were tested against AS particles for ATPase inhibitory activity. IF_1 was preincubated for 15 min at 30 °C with AS particles (0.025 mg of protein) in 0.25 M sucrose, 10 mM MOPS, 0.5 mM ATP, and 0.5 mM $MgSO_4$, pH 6.5, in a final volume of 0.25 mL; the remaining ATPase activity was assayed at pH 8 as described under Materials and Methods.

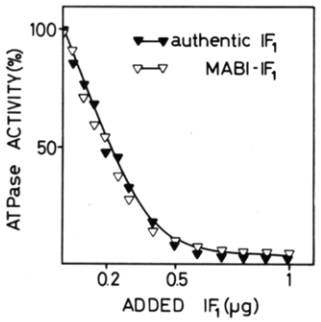

FIGURE 3: Titration curves of AS particles with native IF_1 and MABI-IF_1. AS particles (25 μg) were incubated in the dark in the presence of increasing amounts of native (▼▼) or MABI-IF_1 (▽▽, 1 MABI/IF_1) and the residual ATPase activity was measured.

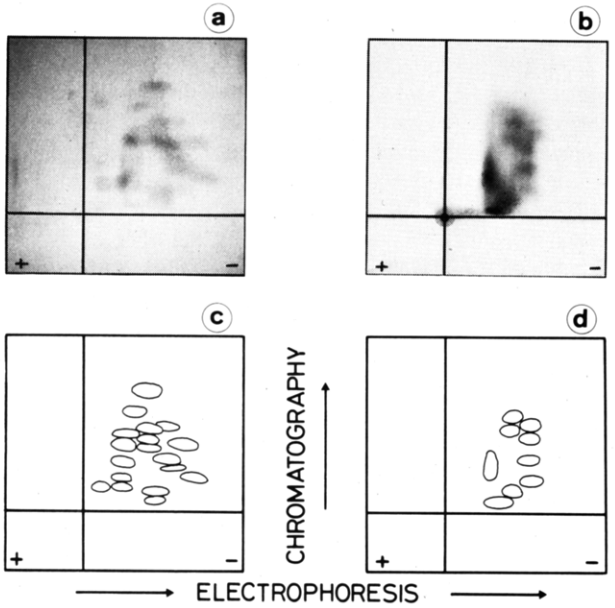

FIGURE 4: Tryptic peptide mapping of (^{14}C)MABI-IF_1. Digestion by trypsin and separation of peptides by electrophoresis followed by chromatography were as described under Materials and Methods. Peptides were visualized by ninhydrin staining and by autoradiography. A photograph (plate a), and a representative drawing (plate c) of the ninhydrin stained spots together with a photograph (plate b) and a representative drawing (plate d) of the autoradiogram are presented.

by PITC, a reagent which, like MABI, binds to lysine residues in IF_1 (Klein et al., 1980).

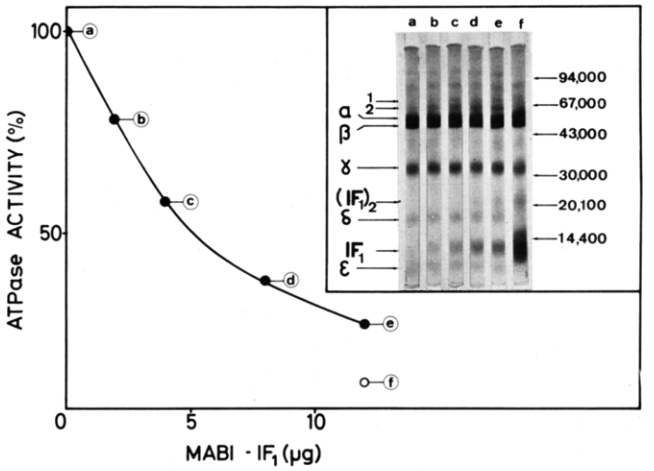

FIGURE 5: Cross-linking of MABI-IF_1 to F_1. To 75 μg of F_1 were added the following amounts of MABI-IF_1: (a) none, (b) 2 μg, (c) 4 μg, (d) 8 μg, (e) 12 μg, (f) 12 μg plus 38 μg of native IF_1, in a final volume of 0.125 mL. Ten microliters was withdrawn for determination of the residual ATPase activity. The remaining was photoirradiated as described under Materials and Methods and then submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis. The figure shows the inhibition of the ATPase activity, and, in the insert, the corresponding gel profiles.

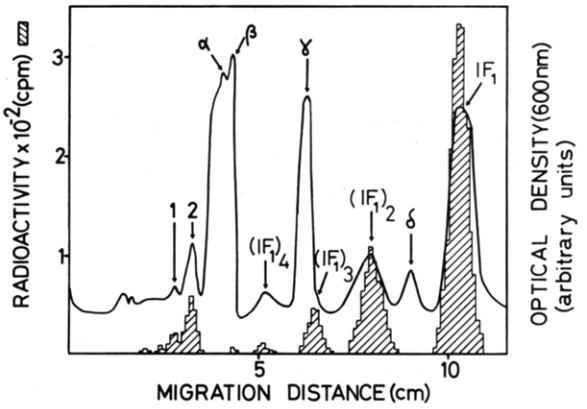

FIGURE 6: Radioactivity profile of the cross-linked (^{14}C)MABI-IF_1–F_1 complex. F_1 (75 μg of protein) was added to (^{14}C)MABI-IF_1 (13 μg of protein). The final volume was 0.125 mL. The mixture was photoirradiated as described under Materials and Methods and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The ^{14}C radioactivity profile corresponds to the shadowed area and the densitometric trace at 600 nm to the plain line.

Photo-Cross-Linking of Isolated F_1 with (^{14}C)MABI-IF_1. As shown in Figure 5, ATPase activity of isolated F_1 steadily decreased upon incubation in the dark with increasing concentrations of (^{14}C)MABI-IF_1. Aliquots corresponding to different extents of F_1 inhibition were photoirradiated with UV light as described under Materials and Methods and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Beside the bands corresponding to F_1 peptides and to the monomeric and dimeric forms of IF_1 found in the control, two new bands, referred to as 1 and 2, were revealed by Coomassie Blue staining in the photoirradiated samples. They became more pronounced as the concentration of MABI-IF_1 was increased (gels a–e, Figure 5). Their molecular weights were approximately 71 000 and 61 000 respectively. When F_1 was incubated with MABI-IF_1 together with an excess of native IF_1, bands 1 and 2 no longer accumulated (gel f, Figure 5).

As shown in Figure 6, bands 1 and 2 are radiolabeled and therefore correspond to complexes containing (^{14}C)MABI-IF_1. Radioactive labeling permitted their differentiation from other high molecular products that are formed by UV-light-induced

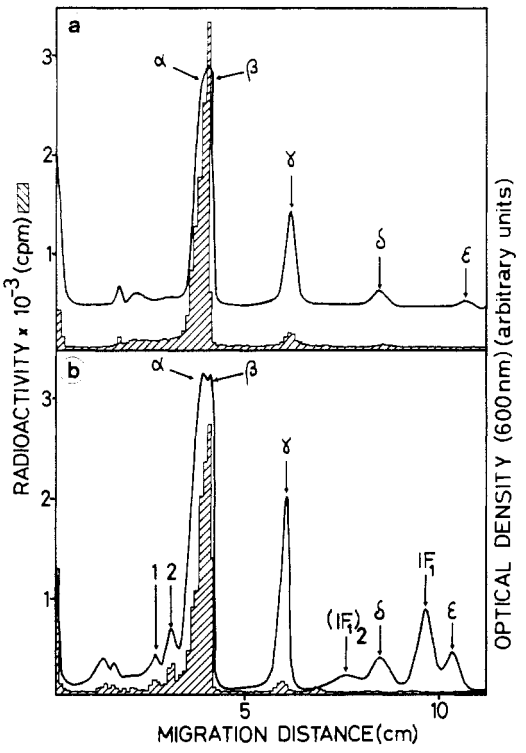

FIGURE 7: (^{14}C)DCCD labeling of photoirradiated F_1 and F_1–MABI-IF_1 complex. F_1 (a) and MABI-IF_1-F_1 complex (b) were photoirradiated as described under Materials and Methods. The amount of F_1 used was 75 μg of protein and that of MABI-IF_1 4.5 μg of protein. (^{14}C)DCCD (100 μM) was then added and allowed to react for 30 min at 30 °C, resulting in incorporation of 1.2–1.4 mol of (^{14}C)DCCD per mol of F_1 or MABI-IF_1–F_1. Free (^{14}C)DCCD was removed by filtration on a Sephadex G-50 column (Penefsky, 1977), and the samples were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The ^{14}C radioactivity profile (cross-hatched area) is shown under the densitometric trace at 600 nm.

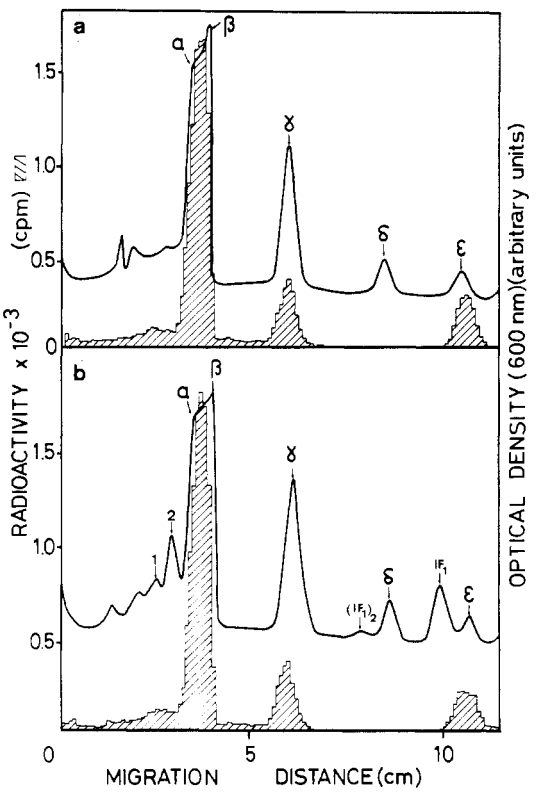

FIGURE 8: (^{14}C)NEM labeling of photoirradiated F_1 and F_1–MABI-IF_1 complex F_1 (a) and MABI-IF_1–F_1 complex (b) were photoirradiated exactly as in Figure 7. To the samples 3 mM (^{14}C)NEM and 1% (w/v) $NaDodSO_4$ were added and allowed to react for 20 min at 30 °C. Samples were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The ^{14}C radioactivity profile (cross-hatched area) is shown under the densitometric trace at 600 nm.

cross-linking of F_1 subunits. Radioactive products whose molecular weights are multiple of that of (^{14}C)MABI-IF_1 also accumulated. Since they were also formed by irradiation of (^{14}C)MABI-IF_1 alone, in the absence of F_1, they were identified as dimer, trimer, and possibly tetramer of (^{14}C)-MABI-IF_1, indicating the tendency of IF_1 to self-association. It must be stressed that oligomers beyond the tetramer were not generated in significant amounts in our experimental conditions. This excludes the contribution of high molecular weight oligomers of (^{14}C)MABI-IF_1 to the cross-linked products 1 and 2 that accumulate after photoirradiation of a mixture of F_1 and (^{14}C)MABI-IF_1.

Identification of Cross-Linked Products 1 and 2 by Specific Labeling of F_1 Subunits. The most plausible candidates to fit the molecular weights of products 1 and 2 are $\alpha(IF_1)_2$, $\beta(IF_1)_2$, $\alpha(IF_1)$ or $\beta(IF_1)$, where $(IF_1)_2$ refers to a dimeric form of IF_1. The approach used to identify unambiguously products 1 and 2 was based on the fact that the α subunit of F_1, but not the β subunit, possesses SH groups and thus can bind alkylating reagents (Senior, 1975) and that the β in isolated F_1 can specifically bind DCCD (Pougeois et al., 1979). Consequently, if either product 1 or 2 is labeled by (^{14}C)NEM, it will be concluded that it contains subunit α; conversely, labeling by (^{14}C)DCCD will specify the presence of subunit β. Such a strategy was successfully used for the identification of subunits after cross-linking of F_1 alone, in a study of the arrangement of F_1 subunits (Satre et al., 1976). It is noteworthy that DCCD and NEM do not react with IF_1.

As shown in Figure 7b, both bands 1 and 2 were labeled with (^{14}C)DCCD, which indicates that both contain subunit β. They can therefore be identified with $\beta(IF_1)_2$ and $\beta(IF_1)$, respectively. In contrast to (^{14}C)DCCD labeling, labeling by (^{14}C)NEM yielded virtually the same radioactivity pattern for F_1 alone and F_1 cross-linked to MABI-IF_1 (Figure 8); the very slight increase of radioactivity in the region where products 1 and 2 are located may suggest the formation of a very small proportion of $\alpha(IF_1)$ product. These data strongly suggest that IF_1 binds essentially to the β subunit of F_1.

Effect of AMPPNP, Quercetin, and Aurovertin on Cross-Linking of MABI-IF_1 to F_1. AMPPNP, a nonhydrolyzable analogue of ATP (Garrett & Penefsky, 1975), and quercetin (Lang & Racker, 1974) share certain properties with IF_1. In fact they behave apparently as unidirectional inhibitors: they inhibit both soluble and particulate mitochondrial ATPase and have no effect on oxidative phosphorylation in submitochondrial particles. Aurovertin, a specific ATPase inhibitor, binds to the β subunit of F_1 (Verschoor et al., 1977).

As shown in Table I, AMPPNP and quercetin markedly reduced the photoinduced covalent binding of (^{14}C)MABI-IF_1 to F_1. This strongly suggests that quercetin competes with MABI-IF_1 for binding to the β subunit of F_1. The effect of AMPPNP is in agreement with previous data obtained with biosynthetically labeled IF_1 showing that AMPPNP cannot substitute for ATP to promote the binding of IF_1 to AS particles (Klein et al., 1977). The fact that aurovertin has no effect on the binding of (^{14}C)MABI-IF_1 to F_1 suggests that either the binding sites for aurovertin and IF_1 are different or IF_1 binds more firmly to F_1 than aurovertin does.

Photo-Cross-Linking of Membrane-Bound ATPase by (^{14}C)MABI-IF_1. AS particles supplemented with (^{14}C)-MABI-IF_1 were photoirradiated under UV light and the la-

Table I: Effect of Quercetin, AMPPNP, and Aurovertin on the Formation of Cross-Linked Bands 1 and 2 Containing (^{14}C)MABI-IF$_1$[a]

additions	^{14}C radioactivity in bands 1 and 2 (%)
ATP	100
ATP + quercetin	28
AMPPNP	31
ATP + aurovertin	100

[a] F_1 (100 μg) was incubated in 2 mL of a medium containing 0.25 M sucrose, 10 mM MOPS, and 1 mM $MgCl_2$, pH 6.5, with 25 μg of (^{14}C)MABI-IF$_1$. Where indicated, 0.5 mM ATP, 0.5 mM AMPPNP, 1 mg/mL quercetin, and 10 μM aurovertin were added. After 15 min of incubation in the dark, the F_1-(^{14}C)MABI-IF$_1$ complex was irradiated with UV light and analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis. The region of the gel corresponding to bands 1 and 2 was cut out and digested with H_2O_2, and its radioactivity counted.

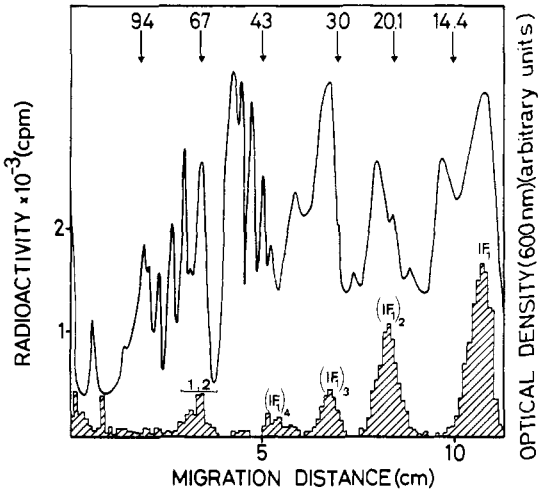

FIGURE 9: Cross-linking of (^{14}C)MABI-IF$_1$ to AS particles. AS particles (200 μg) were incubated with (^{14}C)MABI-IF$_1$ (13 μg) for 15 min in the dark and then irradiated with UV light. The cross-linked sample was treated with 0.1% $NaDodSO_4$ and then analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis. The figure shows the ^{14}C radioactivity profile (cross-hatched area) and the densitometric trace at 600 nm.

beled proteins were separated after lysis of the particles by $NaDodSO_4$-polyacrylamide gel electrophoresis (Figure 9). Beside IF_1 oligomers (bands $(IF_1)_2$, $(IF_1)_3$, $(IF_1)_4$), the peak of radioactivity corresponding to molecular weights in the range of 60000 to 70000 was located at the same place as that observed for the cross-linked products 1 and 2 obtained by reaction of IF_1 with the β subunit of soluble F_1.

Effect of Energization on the Reversible Binding of (^{14}C)MABI-IF$_1$ to MgATP Particles (Dark Reaction). Respiring MgATP submitochondrial particles can readily develop a proton-motive force (Sorgato & Ferguson, 1979; Villiers et al., 1979) of virtually the same value as that found in mitochondria. Kinetic data obtained with MgATP particles were interpreted to mean that IF_1 is released from F_1 upon energization of the mitochondrial membrane (Van de Stadt et al., 1973; Van de Stadt and Van Dam, 1974; Harris et al., 1979). To test the validity of this interpretation, (^{14}C)-MABI-IF$_1$ was used as a reversible ligand of membrane-bound F_1 and its binding affinity was determined under various conditions of activity of the respiratory chain in the absence of photoirradiation. Interaction of added (^{14}C)MABI-IF$_1$ with membrane-bound F_1 in MgATP particles was feasible because most of the particles were inside out (Racker, 1970), which made their membrane-bound F_1 accessible to ligands of the incubation medium. MgATP particles were preincubated with (^{14}C)MABI-IF$_1$ (Table II). Aliquots of the suspension were then incubated with NADH for a few minutes; another incubation was run in the presence of antimycin to inhibit NADH oxidation; and finally a control incubation without NADH was performed for the same period of time. ATPase activity of the incubated particles was measured; then the bound (^{14}C)MABI-IF$_1$ was determined on the sedimented particles after high-speed centrifugation. Incubation with NADH increased the ATPase activity and, in parallel, decreased the amount of bound radioactivity with respect to a control nonsupplemented with NADH. Addition of antimycin to NADH-respiring particles resulted in a decrease of ATPase activity and an increase of bound radioactivity. These data are interpreted as follows. In the control, the bound radioactivity reflects the binding of (^{14}C)MABI-IF$_1$ to F_1 in particles, most likely by exchange with IF_1. The decrease in bound radioactivity which accompanies NADH oxidation is explained by the release of the previously bound (^{14}C)MABI-IF$_1$; this is fully consistent with the concomitant increase in ATPase activity.

Table II: Effect of Energization on Binding of (^{14}C)MABI-IF$_1$ to MgATP Submitochondrial Particles[a]

additions	ATPase activity (μmol of P_i min^{-1} (mg particles)$^{-1}$)	specifically bound (^{14}C)MABI-IF$_1$ (pmol (mg particles)$^{-1}$)
none	0.70	154
NADH	0.92	70
NADH + antimycin A	0.75	107

[a] MgATP particles (2 mg) were preincubated for 15 min in the dark in 2 mL of a medium containing 0.25 M sucrose, 4 mM potassium phosphate, 0.5 mg/mL BSA, 1 mM ADP, and 1 mM $MgCl_2$, pH 7.0 with 10 μg of (^{14}C)MABI-IF$_1$. This was followed by addition, where indicated, of 2 mM NADH and 10 μg of antimycin A. After 0.5-min incubation at 20 °C, 50 μL of the suspension was removed for measurement of the ATPase activity and the remaining suspension centrifuged at top speed in a Sorvall centrifuge at 0 °C. The walls of the tubes were rinsed and the pellets dissolved in 1 mL of formamide at 180 °C. A parallel series of incubations with MgATP particles previously heated at 100 °C for 5 min was carried out to determine the nonspecifically bound (^{14}C)MABI-IF$_1$. The nonspecifically bound radioactivity was subtracted from the total bound radioactivity to calculate the amount of specifically bound (^{14}C)MABI-IF$_1$.

Discussion

MABI was introduced by Ji (1977) as a photosensitive heterobifunctional cross-linking reagent and used to determine the receptor site for concanavalin A in the human erythrocyte membrane. The present work describes the synthesis of a radiolabeled photoactivable derivative of IF_1, (^{14}C)MABI-IF$_1$, and its use to investigate the localization of IF_1 binding sites on F_1 and the modulation of F_1–IF_1 interaction in submitochondrial particles as a function of the energy state of the particles. Critical tests of the biological activity of MABI-IF$_1$ were performed. (^{14}C)MABI-IF$_1$ displayed the same inhibitory potency as native IF_1 on the ATPase activity of F_1 provided that the number of incorporated moles of MABI per mole of IF_1 did not exceed 5. In routine assays, 1–1.5 mol of (^{14}C)MABI was incorporated per mol of IF_1, thus resulting in fully active (^{14}C)MABI-IF$_1$. By specifically labeling the α subunit of F_1 by (^{14}C)NEM (Senior, 1975) and the β subunit by (^{14}C) DCCD (Pougeois et al., 1979), it was possible to

locate unambiguously the IF_1 binding site in the β subunit of F_1. These data corroborate previous ones (Klein et al., 1980) based on a different approach, where IF_1 was first radiolabeled by (^{14}C)PITC and then covalently bound to F_1 by the cross-linking reagents EDAC or EEDQ. The advantage of (^{14}C)-MABI-IF_1 over (^{14}C)PITC-IF_1 is that binding of (^{14}C)-MABI-IF_1 to F_1 can be achieved directly by photoactivation without the help of added cross-linkers. One may add that cross-linking of juxtaposed subunits by reagents like EDAC and EEDQ requires proper alignment of reactive groups in these subunits. Covalent photo-cross-linking of (^{14}C)-MABI-IF_1 with the interacting subunit in F_1 is probably not subject to this steric restriction because the nitrene generated by photoirradiation of (^{14}C)MABI-IF_1 is characterized by a high and unspecific reactivity toward a variety of chemical groups in proteins. Consequently the present data bring strong evidence for specific interaction between IF_1 and the β subunit of F_1. The low yield of photo-cross-linking (about 10%) reported in this paper is probably due to technical limitations. In fact, it is known (Guillory, 1979) that photoaffinity labeling is a low-yield reaction, because of photoproduct inhibition. The stoichiometry of the binding of (^{14}C)MABI-IF_1 to IF_1 was not examined in this paper. It must be recalled that a stoichiometry of 1 was found for the binding of IF_1 (labeled by (^{14}C)PITC) to F_1 (mol/mol) (Klein et al., 1980).

A few examples of the potential use of (^{14}C)MABI-IF_1 are given. Addition of AMPPNP and quercetin prevented the binding of (^{14}C)MABI-IF_1 to F_1. Quercetin may directly compete with IF_1 for binding to the β subunit of F_1. It was previously reported that AMPPNP was inefficient (in contrast to ATP) to promote the binding of biosynthetically radiolabeled (^{14}C)IF_1 to AS particles. This also applies for the binding of (^{14}C)MABI-IF_1 to F_1, which gives further evidence for the similar biological behavior of native IF_1 and MABI-IF_1. (^{14}C)MABI-IF_1 was also used in the absence of light to probe the effect of energization of submitochondrial particles on F_1–IF_1 interaction. In a number of reports, the interaction of IF_1 with membrane-bound F_1 in submitochondrial particles has been assessed on the basis of the inhibition of ATPase activity or ATP synthesis; dissociation of IF_1 from F_1 in submitochondrial particles was postulated to result in an increased maximal velocity of the ATPase activity (Van de Stadt et al., 1973; Van de Stadt & Van Dam, 1974; Ernster et al., 1973) or in a shortening of the lag period which preceeds phosphorylation (Harris et al., 1979). Although a direct relationship between IF_1–F_1 interaction and the modulation of F_1 activity was implicitly recognized in these experiments, direct evidence of changes in binding affinity of IF_1 for F_1 was lacking. The preliminary data obtained with (^{14}C)MABI-IF_1 provide further support to the hypothesis that the energy state of mitochondrial particles indeed modulates the binding affinity of F_1 for IF_1 and that the proton-motive force developed by mitochondrial respiration results in a release of IF_1.

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