Photoinactivation of the Bovine Heart Mitochondrial F_1-ATPase by [^{14}C]Dequalinium Cross-Links Phenylalanine-403 or Phenylalanine-406 of an α Subunit to a Site or Sites Contained within Residues 440–459 of a β Subunit†

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ABSTRACT: Synthesis of [^{14}C[dequalinium, 1,1′-(1,10-[1,10-^{14}C]decanediyl)bis[4-amino-2-methylquinolinium], is described, which photoinactivates the bovine heart mitochondrial F_1-ATPase (MF_1). Maximal photoinactivation occurs on incorporation of about 1.5 mol of [^{14}C]dequalinium/mol of MF_1. Three radioactive species were resolved when photoinactivated enzyme was submitted to polyacrylamide gel electrophoresis at pH 4.0 in the presence of tetradecyltrimethylammonium bromide, which correspond to the α and β subunits and a cross-linked species with an M_r of 116 000. Fractionation of a tryptic digest of photoinactivated enzyme by high-performance liquid chromatography led to isolation of a radioactive peptide which contains residues 399–420 of a α subunit. Two fragments containing equal amounts of radioactivity were obtained on fractionation of an endoproteinase Asp-N digest of the isolated radioactive tryptic peptide by high-performance liquid chromatography. Amino acid sequence analysis showed that both fragments contained residues 399–408 of the α subunit, but one was missing Phe-α403 and the other was lacking Phe-α406. Fractionation of a cyanogen bromide digest of photoinactivated enzyme followed by trypsin digestion of partially purified cyanogen bromide fragments and fractionation of the resulting radioactive tryptic fragments yielded several radioactive species comprised of residues 399–420 of the α subunit cross-linked to residues 440–459 of the β subunit and a radioactive fragment containing residues 399–420 of the α subunit. Partial sequence analyses of the cross-linked fragments suggest that Phe-α403 and Phe-α406 participate in cross-links, whereas no information was obtained on the site or sites of cross-linking in the β subunit fragment. MF_1 is reversibly inhibited in the dark and photoinactivated in the presence of 4-amino-1-octylquinaldinium. The results obtained suggest that the binding site for dequalinium is at an interface of α and β subunits, but formation of cross-links is not necessary for photoinactivation observed in the presence of dequalinium.

The ATP synthases of energy transducing membranes couple proton electrochemical gradients generated by electron transport processes to condensation of ADP with P_i to form ATP. The membrane-bound enzymes are comprised of an integral membrane protein complex, F_o, and a peripheral membrane protein complex, F_1. Resolution and reconstitution studies have shown that F_o mediates transmembrane proton conduction, whereas F_1 contains the catalytic sites for ATP synthesis (Filligame, 1990). When removed from the membrane in soluble form, F_1 is an ATPase (Senior, 1988; Futai et al., 1989). The bovine heart mitochondrial F_1-ATPase (MF_1)[1] is composed of five gene products, designated α–ϵ, in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. The molecular weight of MF_1, based on the amino acid sequences deduced for its constituent subunits, is 371 000 (Walker et al., 1985).

The F_1-ATPases are reversibly inhibited by a variety of amphiphathic cations. Included among these are the following: substituted phenothiazines (Chazotte et al., 1982; Palatini, 1982), substituted xanthenes (Emaus et al., 1986; Wieker et al., 1987; Bullough et al., 1989a), substituted acridines (Laikind & Allison, 1983; Bullough et al., 1989a), the bee venom peptide, melittin, and synthetic peptides corresponding to the presequence of yeast cytochrome oxidase subunit IV (Bullough et al., 1989a). The lipophilic cations are either noncompetitive (Palatini, 1982; Adade et al., 1984; Bullough et al., 1989a), mixed (Bullough et al., 1989a), or uncompetitive (Tuena de Gomez-Puyou et al., 1977; Laikind & Allison, 1983; Wieker et al., 1987) inhibitors of the ATPase when examined by steady-state kinetics, indicating that they do not bind to the catalytic site. The ATPase activity of MF_1 is inactivated by the aziridinium generated from quinacrine mustard (Laikind & Allison, 1983; Bullough et al., 1989b). It has been shown that inactivation by quinacrine mustard is accompanied by esterification of one or any one of the carboxylic acid side chains in a highly conserved region of the β subunit with the sequence: $_{394}$DELSEED$_{401}$. Amphipathic cations which are reversible inhibitors of the ATPase have been shown to protect the enzyme against inactivation by quinacrine mustard in order of their experimentally determined K_i's (Bullough et al., 1989b). This suggests that the DELSEED segment of the β subunit might provide part of the binding site for inhibitory amphipathic cations in general.

Dequalinium, 1,1′-(1,10-decanediyl)bis[4-amino-2-methylquinolinium], inhibits MF_1 noncompetitively in the absence of light with a K_i of 7.5 μM (Bullough et al., 1989a). Irradiation of MF_1 in the presence of dequalinium at 350 nm rapidly inactivates the enzyme (Zhuo & Allison, 1988). By examining the rate of inactivation of MF_1 as a function of

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[1] Abbreviations: MF_1, CF_1, EF_1, TF_1, and YF_1, the F_1-ATPases from bovine heart mitochondria, spinach chloroplasts, *Escherichia coli*, the thermophilic bacterium PS3, and yeast mitochondria, respectively; TDAB, tetradecyltrimethylammonium bromide; MOPS, 4-morpholinepropanesulfonic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography.

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dequalinium concentration, an apparent K_d of 12.5 μM was estimated for reversible binding of the inhibitor to MF_1 prior to covalent modification. We report here the synthesis of dequalinium labeled with ^{14}C and use of the radiolabeled inhibitor to identify the site in MF_1 that is derivatized during photoinactivation.

MATERIALS AND METHODS

Materials. MF_1 was prepared by a previously described modification (Esch & Allison, 1978) of an earlier method (Knowles & Penefsky, 1972a). The enzyme was stored at 4 °C as a suspension in 55% saturated ammonium sulfate (pH 7.0) containing 4 mM ATP and 2 mM EDTA. Enzyme was desalted by passing it through centrifuge columns of Sephadex G-25 as described (Penefsky, 1978). ATPase activity was determined spectrophotometrically by coupling oxidation of NADH by lactate dehydrogenase to pyruvate released from the phosphoenolpyruvate/pyruvate kinase system used to regenerate ATP (Pullman et al., 1960). Dequalinium dichloride, enzymes, biochemicals, and buffer components were purchased from Sigma Chemical Co. Before use, dequalinium dichloride was recrystallized from acetone–water to remove contaminants. Trypsin treated with TPCK was purchased from Worthington. Endoproteinase Asp-N was purchased from Sigma. HPLC solvents were purchased from Fisher.

4-Amino-1-octylquinaldinium was prepared by refluxing equivalent amounts of 1-iodooctane (Aldrich) and 4-aminoquinaldine (Aldrich) in 4-methyl-2-pentanol (Aldrich) for 72 h. Product was purified by cation-exchange HPLC on a SCX 300 column using a gradient of NaCl in triethylamine hydrochloride, pH 6.8, as eluant. The peak containing 4-amino-1-octylquinalinium was desalted by HPLC on a PRP-polymer reversed-phase column which was equilibrated with water and eluted with a gradient of acetonitrile to 90%.

Synthesis of [^{14}C]Dequalinium. [^{14}C]Dequalinium chloride, the ^{14}C of which was ultimately derived from [1,10-^{14}C]sebacic acid, was synthesized as follows. In the first step, [^{14}C]sebacic acid was reduced to [^{14}C]decanediol by modification of a described method (Nystrom & Brown, 1947). To this end, 250 μCi of [^{14}C]sebacic acid (Sigma Chemical Co.) was diluted to 3 cpm/pmol with 89 μmol of sebacoyl chloride (Aldrich) in 1 mL of dry tetrahydrofuran and 2 mL of dry diethyl ether in a 10-mL Pierce Reactivial equipped with a 20-cm condenser. After finding significant amounts of impurities in commericial sebacic acid from several sources, the [^{14}C]sebacic acid, which appeared free of impurities, was diluted with sebacoyl chloride (Aldrich) which was also free of contaminants. To this solution was added 2 mL of 1 M $LiAlH_4$ in diethyl ether (Aldrich) over 10 min with vigorous stirring. The mixture was stirred an additional 45 min, at which time 1 mL of 3.6 M H_2SO_4 was added to decompose excess $LiAlH_4$. The diethyl ether layer was then removed from the aqueous layer with a syringe. The aqueous layer was extracted five times with 1-mL portions of diethyl ether. The extracts and original diethyl ether layer were combined and washed with 1 mL of saturated $NaHCO_3$ and then with 1 mL of saturated NaCl. After washing, 0.5 g of anhydrous Na_2SO_4 was added and the solution was allowed to dry overnight at room temperature in a closed 10-mL Pierce Reactivial. The yield of [^{14}C]decanediol was 89%.

The dried [^{14}C]decanediol was converted to the bistosylate as described (Brandsma, 1988). To the diethyl ether solution of [^{14}C]decanediol was added 300 mg of tosyl chloride (Fluka), and the solution was cooled to 4 °C. Then 0.5 g of freshly crus' ed KOH was slowly added over 5 min, which was followed by vigorous stirring for 1 h at 4 °C, at which time 2 mL of ice water was added to decompose the unreacted tosyl chloride. The organic phase was removed with a syringe. The aqueous phase was then extracted with five 1-mL portions of diethyl ether. The combined extracts and original organic phase were combined and washed with 1 mL of saturated $NaHCO_3$ followed by 1 mL of saturated NaCl. After drying the combined material with 300 mg of $MgSO_4$, diethyl ether was allowed to evaporate in a fume hood. The yield of [^{14}C]-decane–bistosylate was 80%.

To prepare [^{14}C[diiododecane, the [^{14}C]decane–bistosylate from the previous step was refluxed with 600 mg of NaI in 5 mL of acetone in a 10-mL Pierce Reactivial fitted with a 20-cm condenser for 2 h to form [^{14}C]diiododecane. Acetone was then removed by distillation. The residue was suspended in 3 mL of distilled water. The product was then extracted from the suspension with 1-mL portions of diethyl ether until most of the radioactivity was removed. The extracts were combined and concentrated to less than 1 mL by evaporation in a fume hood. The concentrated solution was applied to a 5-mL silica column (Bio Sil A, 200–400 mesh, Bio-Rad) which was packed and eluted with pentane. The radioactive peak that was eluted contained a single radioactive species, [^{14}C]-diiododecane, from which solvent was removed under vacuum. The yield of [^{14}C]diiododecane was 65%.

The [^{14}C]diiododecane from the previous step was refluxed with 90 mg of 4-aminoquinaldine (Sigma) for 72 h in 5 mL of 4-methyl-2-pentanol (Aldrich) as described (Austin et al., 1958). The solid containing product which was formed during reflux was collected by centrifugation and dissolved in 50% ethanol. The resulting solution was submitted to cation-exchange HPLC on an SCX-300 column which was equilibrated with 0.2% triethylamine hydrochloride, pH 6.8, containing 30% methanol and eluted with a gradient of 0–300 mM NaCl in the same buffer. Three peaks of radioactivity eluted from the column. The material in the second peak, which accounted for 68% of the radioactivity recovered, had the same retention time as authentic dequalinium when submitted to HPLC under a variety of conditions. It was concentrated under vacuum, and to remove salt, the concentrate was submitted to HPLC on a PRP-1 polymer reversed-phase column which was equilibrated with water and eluted with a gradient of acetonitrile to 90%. The purified [^{14}C]-dequalinium chloride was stored as a 9.6 mM solution in 50% ethanol. The overall yield of the synthesis was 31%.

Analytical Methods. Each step of the synthesis of [^{14}C]-dequalinium was monitored by TLC (1318 silica gel, Kodak) using diethyl ether or pentane as developing solvent. To detect the positions of radioactive products, the lanes of developed TLC sheets were cut into strips which were counted in 3 mL of scintillation fluid. Protein concentrations were determined with bicinchoninic acid (Pierce Chemical Co.) as described by Smith et al. (1985). Radioactivity was determined by liquid scintillation counting in Liquiscint from National Diagnostics using a Tm Analytic 6795 counter. Automatic Edman degradations were carried out with an Applied Biosynthesis 470A gas-phase sequenator. Peptide separations by HPLC were performed with an Altex Model 332 gradient liquid chromatograph equipped with an Applied Biosystems absorbance detector and a Shimadzu C-R1A Chromatopac integrating recorder. The HPLC columns used were as follows: phenyl and C_{18} reversed-phase (Brownlee, 22 × 0.46 cm, 7-μm particle size); PRP-1 polymer reversed-phase (Hamilton, 30 × 0.78 cm); SCX-300 Å cation exchange (Rainin, 10 × 0.78 cm, 5–12-μm particle size), TSK $G2000_{SW}$

gel permeation (60 × 0.70 cm), and TSK G4000$_{SW}$ gel permeation (30 × 0.75 cm).

Polyacrylamide gel electrophoresis in the presence of TDAB was performed as described by Penin et al. (1984) except that 0.2 M sodium phosphate, pH 4.0, was used rather than 0.2 M sodium phosphate, pH 2.0. Gels were stained with 0.1% Coomassie Blue 250 in 5% acetic acid–40% methanol. Labeled enzyme which was submitted to electrophoresis was prepared in the following manner. After photoinactivating 1.2 mg of MF_1 by 89% with 12 μM [^{14}C]dequalinium at 23 °C in 0.8 mL of 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM Mg^{2+} and 5 mM β-mercaptoethanol, free [^{14}C]dequalinium was removed by passing two 400-μL aliquots of the reaction mixture through 5-mL centrifuge columns containing Sephadex G-50 equilibrated with the same buffer. The eluted protein was precipitated by the addition of solid ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation, dissolved in 200 μL of 6 M guanidinium chloride, pH 7.0, and dialyzed at 23 °C against 1 L of distilled water for 1 h and then for an additional 1 h against 1 L of fresh distilled water. The protein precipitate containing the α, β, and γ subunits (Knowles & Penefsky, 1972) was collected by centrifugation and was dissolved in 0.2 M sodium phosphate, pH 4.0 or pH 6.0, containing 6% TDAB and 100 mM β-mercaptoethanol (inset of Figure 2) or without β-mercaptoethanol (gel slices of Figure 2) and submitted to electrophoresis on a 1.5-mm slab as described by Penin et al. (1984).

Photoinactivation of MF_1 with [^{14}C]Dequalinium and Preparation of Tryptic and Cyanogen Bromide Digests of Labeled Enzyme. Large-scale photoinactivations were carried out in a Rayonet photochemical reactor with a lamp emitting maximally at 350 nm. Each inactivation mixture contained 12 mg of MF_1 in 8 mL of 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM $MgSO_4$, 5 mM β-mercaptoethanol, and 12 μM [^{14}C]dequalinium. When the enzyme was inactivated by 81–88%, solid ammonium sulfate was added to a final concentration of 60% saturation to precipitate the protein from each reaction mixture. After standing at 4 °C for 30 min, ammonium sulfate precipitates were collected by centrifugation.

To prepare a tryptic digest of enzyme inactivated with [^{14}C]-dequalinium, labeled protein from a single inactivation mixture was dissolved in 0.5 mL of 100 mM Tris–H_2SO_4, pH 7.5, containing 4 mM CDTA. The resulting solution was passed consecutively through two 5-mL centrifuge columns of Sephadex G-50 equilibrated with the same buffer. Solid guanidinium chloride and dithiothreitol were added to final concentrations of 6 M and 10 mM, respectively. After 30 min at 23 °C, iodoacetamide was added to a final concentration of 20 mM. After an additional 90 min, the reaction mixture was dialyzed against 2 L of 1 mM HCl at 4 °C for 6 h. Trypsin was added to the dialyzed solution of labeled protein in a weight ratio of substrate to trypsin of 50:1 which was followed by addition of 1 M MOPS, pH 7.5, to a final concentration of 50 mM. Digestion was carried out with stirring at 23 °C for 15 h.

Labeled protein from three inactivation mixtures was pooled for preparation of a cyanogen bromide digest. The resulting ammonium sulfate precipitate was dissolved in 2 mL of 6 M guanidinium chloride, pH 7.0. This solution was dialyzed against 1 L of deionized water at 23 °C for 1 h with one change to precipitate the α, β, and γ subunits (Knowles & Penefsky, 1972b). The protein precipitate was collected by centrifugation and was dissolved in 3 mL of 70% formic acid. After adding 920 mg of CNBr, the resulting reaction mixture was incubated at 23 °C with continuous stirring for 16 h, at which time it was submitted to high-performance gel permeation chromatography on a TSK G2000$_{SW}$ column.

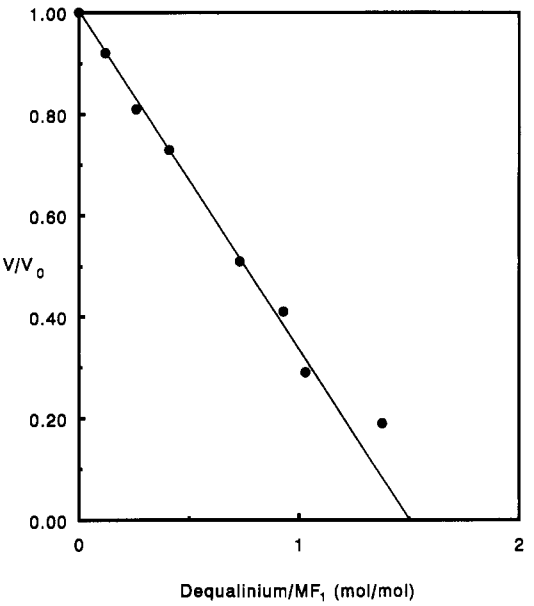

FIGURE 1: Incorporation of ^{14}C during inactivation of MF_1 with [^{14}C]dequalinium. MF_1, 8 mL of a 1 mg/mL solution in 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM $MgSO_4$ and 6 mM β-mercaptoethanol, was photoinactivated with 14 μM dequalinium chloride in the photochemical reactor. At 5, 10, 15, 20, 30, 40, 60, and 90 min., 0.9-mL samples of the reaction mixture were withdrawn. The ATPase activity of 1-μL subsamples was determined. The remaining protein was precipitated by adding solid ammonium sulfate to 55% saturation. The protein suspensions in ammonium sulfate were kept on ice until just before analysis, when they were collected by centrifugation and dissolved in 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM $MgSO_4$. To ensure removal of noncovalently bound radioactive reagent, the redissolved samples were then submitted to gel permeation HPLC on a TSK G-4000$_{SW}$ column which was equilibrated and eluted with the same buffer. The radioactivity in the gel-filtered samples was determined by liquid scintillation counting. The protein concentration of the samples was determined with bicinchoninic acid (Smith et al., 1985).

RESULTS

Covalent Incorporation of ^{14}C during Inactivation of MF_1 with [^{14}C]Dequalinium. To determine incorporation of label during inactivation of MF_1 with [^{14}C]dequalinium, 4.58 μM MF_1 was irradiated at 350 nm in the presence of 14 μM dequalinium in 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM $MgSO_4$ and 6 mM β-mercaptoethanol. MF_1 is more stable in 50 mM sodium pyrophosphate, pH 8.0, containing 3 mM Mg^{2+}, than in 50 mM Tris-HCl or triethanolamine–H_2SO_4 at pH 8.0. Therefore, it was the buffer of choice for these experiments. The initial rate of photoinactivation of MF_1 by dequalinium is about the same in the three buffers. The β-mercaptoethanol was added as a radical scavenger to minimize nonspecific labeling. The amount of radioactivity incorporated per mole of enzyme was determined from samples which were removed with time after initiating photolysis. A plot of the fractional activity remaining against the moles of [^{14}C]dequalinium incorporated per mole of MF_1 is shown in Figure 1. It is clear that, on 82% inactivation of MF_1 with [^{14}C]dequalinium, about 1.4 mol of reagent are incorporated per mole of enzyme. The least squares straight line of the data extrapolates to incorporation of about 1.5 mol of [^{14}C]dequalinium/mol of enzyme on 100% inactivation.

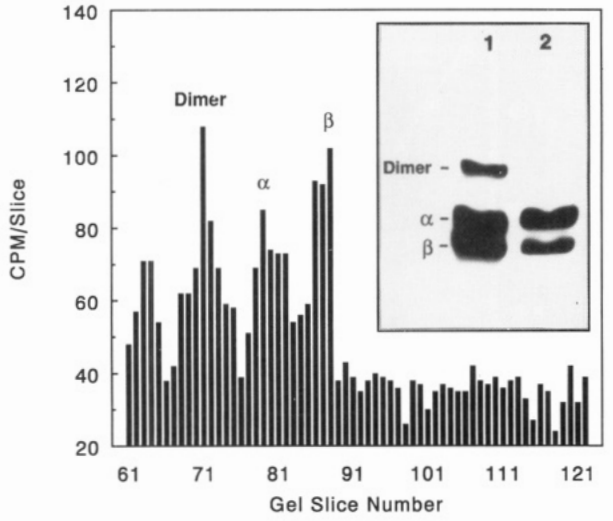

FIGURE 2: TDAB–polyacrylamide gel electrophoresis at pH 4.0 of the α, β, and γ subunits isolated from MF_1 inactivated with [^{14}C]-dequalinium. The preparation of samples submitted to electrophoresis is described in detail under Materials and Methods. Main illustration: 100 μg of the α, β, and γ subunits prepared from MF_1 after inactivation with [^{14}C]dequalinium was submitted to electrophoresis in the presence of TDAB at pH 4.0 without treatment with β-mercaptoethanol. The gel was cut into 1-mm slices which were digested with 30% H_2O_2–14 M NH_4OH (59:5) in scintillation vials for 16 h at 37 °C. Scintillation fluid was then added, and the samples were counted. Inset: Gel stained with Coomassie Blue 250 after submitting the α, β, and γ subunits of MF_1 to electrophoresis at pH 4.0 in the presence of TDAB. Lane 1, 100 μg of the α, β, and γ subunits prepared from MF_1 after inactivation with [^{14}C]dequalinium and treated with 100 mM β-mercaptoethanol; lane 2, 50 μg of the α, β, and γ subunits prepared from native MF_1 and treated with 100 mM β-mercaptoethanol. Standards are not shown.

To ensure complete removal of free [^{14}C]dequalinium in the labeling experiment described above, enzyme samples were precipitated with ammonium sulfate. The precipitates were collected by centrifugation, dissolved in 50 mM sodium pyrophosphate, pH 8.0, and submitted to high-performance gel permeation chromatography on a TSK G-4000$_{SW}$ column which was equilibrated and eluted with the same buffer. Free [^{14}C]dequalinium was well removed from the protein peak in the effluents of these columns. Samples of enzyme photoinactivated by 8–82% with [^{14}C]dequalinium or by 88% with nonradioactive dequalinium eluted from the column in a single, symmetrical peak with the same retention time as unmodified, active enzyme. Therefore, inactivation of MF_1 induced by irradiation in the presence of dequalinium is not caused by dissociation of the modified enzyme.

Cross-Linking Observed on Submitting MF_1 Inactivated with [^{14}C]Dequalinium to Polyacrylamide Gel Electrophoresis in the Presence of TDAB. Samples of the subunit mixtures obtained from photoinactivated and native enzyme were submitted to electrophoresis at pH 4.0 in the presence of TDAB, as adapted from Penin et al. (1984), with and without prior incubation with 100 mM β-mercaptoethanol. Whereas four radioactive bands were resolved when the untreated sample was electrophoresed (Figure 2), three bands staining with Coomassie Blue (inset of Figure 2) were resolved when the treated sample was electrophoresed. In each case, a protein species with an M_r of about 116 000 was resolved in the lane containing enzyme which had been photoinactivated in the presence of dequalinium. The relative amounts of recovered radioactivity distributed in the cross-linked species, α subunit, and β subunit determined from counting slices of the gel shown in the inset were respectively 22%, 55%, and 23%. For the photoinactivated sample which had not been treated with β-mercaptoethanol prior to electrophoresis the relative amounts of radioactivity distributed over the cross-linked species of M_r >120 000, the cross-linked species of M_r about 116 000, α subunit, and β subunit were respectively 12%, 34%, 27%, and 27%. Since the cross-linked species of higher M_r was not observed in the presence of β-mercaptoethanol, but was observed in a control mixture of α, β, and γ subunits obtained from underivatized MF_1, it probably represents α–α dimers cross-linked by disulfide bonds. The γ subunit ran off the gels when electrophoresis was performed at pH 4.0, whereas it remained on gels electrophoresed at pH 6.0. The distribution of radioactivity in resolved species for a sample treated with β-mercaptoethanol and electrophoresed at pH 6.0 in the presence of TDAB was as follows: cross-linked species of M_r of about 116 000, 32%; α subunit, 35%; β subunit, 24%, and γ subunit, 8%. Very little radioactivity was covalently bound to the δ and ϵ subunits which remained soluble after dialysis of photoinactivated enzyme denatured with guanidinium chloride. It is important to note that cross-linking was not observed when enzyme photoinactivated with dequalinium was submitted to SDS–PAGE at pH 8.8, indicating that the cross-links are labile under alkaline conditions.

Reversible Inhibition and Photoinactivation of MF_1 with 4-Methyl-1-octylquinaldinium. Since cross-linking accompanies photoinactivation of MF_1 with dequalinium, it was pertinent to examine the effects of an alkylmonoquinaldinium on the enzyme. It was found that 4-methyl-1-octylquinaldinium reversibly inhibitis MF_1 with an $I_{0.5}$ value of 48 μM at pH 7.5. The $I_{0.5}$ value for dequalinium under the same conditions is 12 μM (Zhuo & Allison, 1988). The ATPase was photoinactivated in the presence of 80 μM 4-methyl-1-octylquinaldinium with a pseudo-first-order rate constant of 7.3×10^{-2} min^{-1} at pH 7.5 and 23 °C. Photoinactivation of the enzyme with 20 μM dequalinium under the same conditions proceeds with a pseudo-first-order rate constant of 5.9×10^{-2} min^{-1}. Therefore, when the alkylmono- and alkylbisquinaldiniums are present at concentrations 1.6-fold greater than their respective $I_{0.5}$ values, the rate of photoinactivation of MF_1 is approximately the same. It is clear from these results that cross-links need not form to observe photoinactivation of MF_1 in the presence of dequalinium.

Isolation of the Major Tryptic Peptide Derived from MF_1 Photoinactivated with [^{14}C]Dequalinium and Identification of Modified Residues in It. Figure 3 summarizes the series of HPLC steps used to purify the major radioactive peptide present in a tryptic digest which was prepared from MF_1 inactivated with [^{14}C]dequalinium described under Materials and Methods. In the first step, a substantial peak of radioactivity, designated T-P_1 in Figure 3, eluted from the phenyl reversed-phase column, which was identified to be [^{14}C]-dequalinium which was not removed from the enzyme in the steps preceding digestion with trypsin. When submitted to reversed-phase HPLC on the phenyl reversed-phase column with the gradient used to resolve the tryptic digest, authentic [^{14}C]dequalinium eluted in 90% yield. Using this value and the portion of the radioactivity eluting in peak T-P_1 to correct for the amount of free [^{14}C]dequalinium in the tryptic digest fractionated, it is estimated that the radioactive material in peak T-P_2 accounts for 37% of the peptide-bound radioactivity applied to the column.

The radioactive material in peak T-P_2 was further fractionated by consecutive HPLC steps. The first employed chromatography on the SCX-300 cation-exchange column, which was followed by chromatography on a C_{18} reversed-

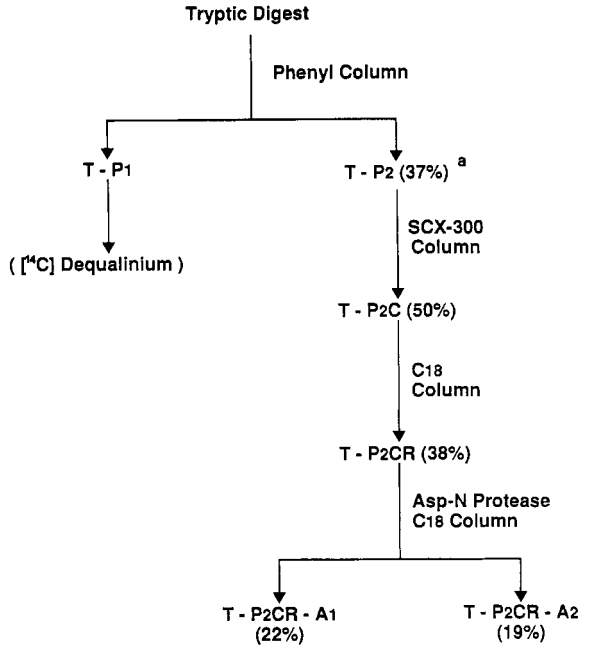

FIGURE 3: Scheme summarizing purification of the major radioactive peptide in a tryptic digest of MF_1 inactivated with [^{14}C]dequalinium and peptides derived from it. The tryptic digest, prepared from MF_1 photoinactivated with [^{14}C]dequalinium described under Materials and Methods, was submitted to the fractionation scheme outlined. The phenyl reversed-phase column was equilibrated with 12 mM HCl and was eluted with a gradient (0–50%) of acetonitrile in 12 mM HCl. The SCX-300 column was equilibrated with 10 mM acetic acid and eluted with a linear gradient of 0–270 mM NaCl in 10 mM acetic acid containing 54% acetonitrile. The C_{18} reversed-phase columns were equilibrated with 12 mM HCl and eluted with linear gradients of acetonitrile in 12 mM HCl. The percentages in parentheses represent yields of radioactivity obtained for each column step. [a] This value represents the yield of peptide-bound radioactivity in the effluent after correcting for free [^{14}C]dequalinium.

phase column. The yield of radioactivity recovered in peak T-P_2C of the cation-exchange column accounts for 50% of that applied. The peak fractions containing the majority of the radioactive material recovered from the C_{18} column, designated T-P_2CR in Figure 3, account for 38% of the radioactivity applied. Assuming that maximal photoinactivation occurs with the incorporation of 1 mol of [^{14}C]dequalinium/mol of MF_1, the material in peak T-P_2CR was isolated in an overall yield of 8%. A portion of this material was submitted to automatic sequence analysis.

The repetitive yields obtained in the sequence analysis of this peptide in peak T-P_2CR are shown in Figure 4A. It is clear that the sequence obtained corresponds to residues 399–420 of the α subunit. Very little radioactivity was associated with that fraction of the Pth-amino acids obtained during the degradation which was submitted to liquid scintillation counting. However, after completion of the Edman degradation, the trifluoroacetic acid-treated glass fiber filter coated with polybrene, on which the peptide was immobilized, was found to contain most of the radioactivity originally applied. This indicates that the Ptc derivative of the amino acid residue or residues derivatized with [^{14}C]dequalinium is not dissolved by *n*-butyl chloride, which is used to remove Ptc-amino acids after the cleavage step. The yields of Pth-amino acids obtained at cycles 2 (V), 5 (F), and 8 (F) are unusually low, suggesting that these residues might have been partially derivatized with [^{14}C]dequalinium.

To provide more information on the location of the label, peptide T-P_2CR was digested with endoproteinase Asp-N. The resulting digest was submitted to HPLC on the C_{18}

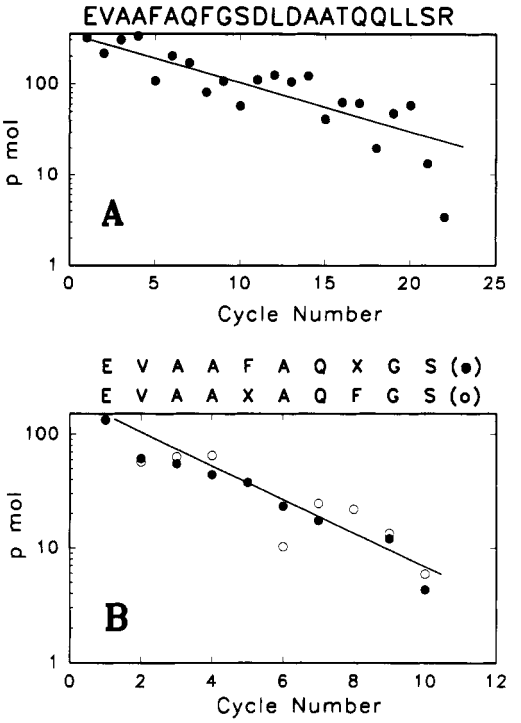

FIGURE 4: Repetitive yields of Pth-amino acids released during automatic Edman degradation of trypic peptide T-P_2CR, T-P_2CR-A_1, and T-P_2CR-A_2. (A) The sequence of T-P_2CR (●). (B) The sequence of T-P_2CR-A_1 (○); the sequence of T-P_2CR-A_2 (●).

reversed-phase column which resolved two radioactive peptides, designated T-P_2CR-A_1 and T-P_2CR-A_2 in Figure 3, in which the ratio of radioactivity was 1.0:0.9. Each peptide was submitted to automatic sequence analysis, the results of which are presented in Figure 4B as semilogarithmic plots of repetitive yields. A blank cycle in which no Pth-amino acid was detected was present in the automatic sequence of each peptide. For peptide T-P_2CR-A_1, the blank cycle corresponds to Phe-α403, whereas that for peptide T-P_2CR-A_2 corresponds to Phe-α406. The two radioactive peptides, T-P_2CR-A_1 and T-P_2CR-A_2, appear to have resulted from the same endoproteinase Asp-N cleavage of the parent tryptic peptide, T-P_2-CR. The relative retentions of the two peptides on the C_{18} reversed-phase column appear to depend on whether Phe-α403 or Phe-α406 is derivatized with [^{14}C]dequalinium. Again, very little radioactivity was found in the samples of the Pth-amino acids which were counted. Also, the trifluoroacetic acid-treated glass fiber filters coated with polybrene, on which the peptides were bound in the sequenator, contained most of the radioactivity originally applied, after sequencing was completed. This also indicates that the Ptc derivative of phenylalanine labeled with [^{14}C]dequalinium is not dissolved by *n*-butyl chloride.

Isolation and Analysis of Radioactive Cyanogen Bromide–Tryptic Peptides after Photoinactivating MF_1 with [^{14}C]-Dequalinium. Given that peptide T-P_2CR was isolated in an overall yield of 8% and that substantial cross-linking was observed when enzyme photoinactivated with [^{14}C]dequalinium was submitted to gel electrophoresis, another approach was taken to obtain information on the site or sites derivatized when MF_1 is photoinactivated. To this end, a mixture of α, β, and γ subunits prepared from MF_1 which had been inactivated with [^{14}C]dequalinium was digested with cyanogen bromide as described under Materials and Methods. The resulting digest was fractionated by high-performance gel permeation chromatography on a column of TSK G-2000$_{SW}$ to yield three peaks of radioactivity which are designated CB_1,

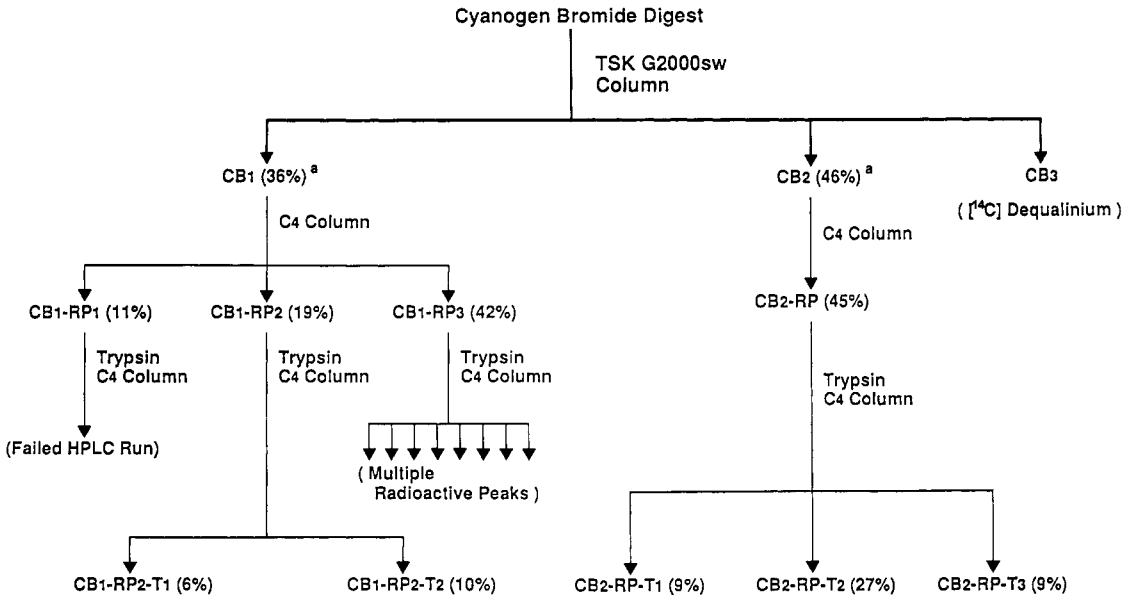

FIGURE 5: Scheme summarizing the purification of radioactive cyanogen bromide–tryptic fragments derived from MF_1 photoinactivated with [^{14}C]dequalinium. The cyanogen bromide digest of MF_1 photoinactivated with [^{14}C]dequalinium described under Materials and Methods. was split into seven 0.5-mL aliquots which were chromatographed on a TSK $G2000_{SW}$ column that was equilibrated and eluted with 30% formic acid. The homologous peaks in the effluents were combined and submitted to HPLC on a C_4 column using a step gradient from 25% to 45% acetonitrile in which the acetonitrile concentration was increased in 0.5% increments at 10-min intervals. After removing acetonitrile in a SpeedVac, 25 μg of trypsin was added to these samples. The pH was then adjusted to 7.8 by addition of 1% NH_4HCO_3 and digestion was allowed to proceed for 1 h at 23 °C, at which time the samples were submitted to HPLC on C_4 reversed-phase columns using a linear gradient of 15–45% acetonitrile in 12 mM HCl. The percentages in parentheses represent yields of radioactivity obtained for each step of the purification. [a] These values represent yields of peptide-bound radioactivity obtained after correcting for free [^{14}C]dequalinium in the effluent.

CB_2, and CB_3 in Figure 5. Peak CB_3 was found to contain free [^{14}C]dequalinium which was not removed from the enzyme prior to digestion with cyanogen bromide. After correcting for free [^{14}C]dequalinium present in the cyanogen bromide digest, the radioactive materials in peaks CB_1 and CB_2 are estimated to account for 36% and 46%, respectively, of the peptide-bound radioactivity applied to the column. The radioactive materials in peaks CB_1 and CB_2 were submitted separately to reversed-phase HPLC on a C_4 column, and the radioactive peptides in the resolved peaks were digested with trypsin. The tryptic digests were then fractionated by HPLC on a C_4 reversed-phase column. The radioactive fractions that were resolved are summarized in Figure 5 along with their corresponding yields.

Three radioactive peptides, designated CB_2-RP-T_1, CB_2-RP-T_2, and CB_2-RP-T_3, were resolved from the tryptic digest of CB_2RP in sufficient purity for sequence analysis. A portion of each was submitted to automatic Edman degradation. Figure 6 illustrates that two Pth-amino acids were revealed in each of eight cycles of sequence analysis of the material in peak CB_2-RP-T_2. Two sequences were deduced from these results, one corresponding to residues 399–406 of the α subunit and the other corresponding to residues 440–447 of the β subunit. The nearly equivalent release of two Pth-amino acids at all but two steps of the Edman degradation suggests that the two peptide fragments might be cross-linked. The following observations support this contention. Ten cycles of automatic Edman degradation of the material in the peak fraction of CB_2-RP-T_1 revealed nearly equivalent release of two Pth-amino acids in each cycle, with the deduced sequences corresponding to residues 399–408 of the α subunit and residues 440–449 of the β subunit. In contrast, when the material in CB_2-RP-T_3 was submitted to 10 cycles of automatic Edman degradation, the sequence corresponding to residues 399–408 of the α subunit was revealed, but but Pth-amino acids corresponding to residues 440–449 of the β subunit were not detected. The observation that a cross-linked species with

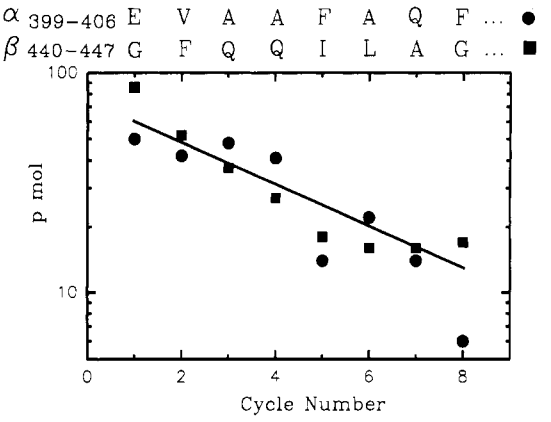

FIGURE 6: Repetitive yields of Pth-amino acids released during automatic Edman degradation of peptide CB_2-RP-T_1. Residues α399–406 (●); residues β440–447 (■).

M_r of about 116 000 was observed when MF_1 which had been photoinactivated with [^{14}C]dequalinium was submitted to polyacrylamide gel electrophoresis at pH 4.0, but not at pH 8.8, suggests that the cross-link is unstable under alkaline conditions. Therefore, the remaining material in peaks CB_2-RP-T_1 and CB_2-RP-T_2 were combined, dried, incubated with 100 mM NH_4HCO_3, pH 7.8, for 15 h at 23 °C, and resubmitted to reversed-phase HPLC on a C_4 column under the conditions used to resolve them originally. On rechromatography, 30% of the radioactivity eluted in the position of peak CB_2-RP-T_3, whereas the remainder eluted in the original positions of peak CB_2-RP-T_1 and CB_2-RP-T_2. This suggests that the slightly alkaline conditions partially cleaved the cross-link, thus releasing the unlabeled β subunit constituent from the labeled α subunit constituent. The low yields of Pth-Phe at cycles 5 and 8 of Figure 6 suggest that peak CB_2-RT-T_2 is composed of two cross-linked peptides, one with Phe-α403 and the other with Phe-α406 cross-linked to unknown side chains within residues 440–459 of the β subunit.

The material in peak CB_1 was fractionated into several peaks of radioactivity when submitted to reversed-phase HPLC which were combined in three pools, designated CB_1-RP_1, CB_1-RP_2, and CB_1-RP_3. The radioactive material in peak CB_1-RP_1 eluted with the same retention time as peak CB_2-RP. After digestion with trypsin, it was lost on a failed HPLC run. The material in peak CB_1-RP_2 was digested with trypsin. The resulting digest was submitted to reversed-phase HPLC on a C_4 column which resolved two sharp peaks of radioactivity, designated CB_1-RP_2-T_1 and CB_1-RP_2-T_2, which were submitted to three cycles of automatic Edman degradation. Sequence analysis revealed that each was comprised of two peptides with the amino-terminal sequences corresponding to residues 399–401 of the α subunit and residues 440–442 of the β subunit, suggesting that they are also cross-linked and structurally related, if not identical to peptides CB_2-RP-T_1 and CB_2-RP-T_2.

After digestion with trypsin, the material in CB_1-RP_3 was fractionated into at least eight peaks of radioactivity, which were resolved when submitted to reversed-phase HPLC on a C_4 column, none of which was present in sufficient amount or purity for amino acid sequence analysis. One of the resolved radioactive peptides had the same retention time as peak CB_1-RP_2-T_1. Of the remaining peaks of radioactivity, one eluted considerably before, whereas the others eluted considerably after, peak CB_1-RP_2-T_1 when submitted to HPLC under the same conditions. The labeled peptides contained in these peaks might have arisen from incomplete cleavages at methionine residues or from nonspecific labeling.

Assuming that modification of 1 mol of a single site/mol of MF_1 with [^{14}C]dequalinium is sufficient for maximal photoinactivation, the sum of the radioactivity in the cyanogen bromide–tryptic peptides which were sequenced reflects an overall yield of 7%. Considering the number of HPLC steps involved in obtaining these peptides, each of which provided much less than quantitative recovery of radioactivity, the low overall yields of peptides obtained from the tryptic digest (8%) and cyanogen bromide–tryptic digests (7%) are not unexpected. All radioactive peptides which were isolated in sufficient amounts for sequence analysis contained either residues 399–420 of the α subunit cross-linked to residues 440–459 of the β subunit, or the α subunit fragment by itself. Two factors probably contribute to the failure to obtain cross-linked peptides in sufficient yield for amino acid sequence analysis with the fractionation procedure used to resolve radioactive peptides in the tryptic digest. Given the demonstrated lability of cross-linked peptides under slightly alkaline conditions, significant cleavage of cross-links might have occurred during carboxamidomethylation prior to tryptic digestion and during digestion, both of which were carried out at pH 7.8. It has also been shown in pilot runs, which are not illustrated, that partially purified, cross-linked peptides in cyanogen bromide–tryptic digests were eluted from the phenyl and C_{18} reversed-phase columns in very low yield. Both columns were used to fractionate the tryptic digest of MF_1 derivatized with [^{14}C]dequalinium. The isolation of radioactive peptides with different chromatographic properties, yet with the same apparent amino acid sequence, probably reflects deriviatization at Phe-α403 or Phe-α406, the homoserine lactone–homoserine equilibirum, and perhaps, heterogeneity of cross–linking sites in the β subunit.

DISCUSSION

The results presented show that photoinactivation of MF_1 by [^{14}C]dequalinium is accompanied by mutually exclusive derivatization of Phe-α403 and Phe-α406 and partial cross-linking of the derivatized phenylalanine residues to a side chain or side chains within residues 440–459 in a neighboring β subunit. The interpretation of these findings which we prefer is that derivatization of Phe-403 or Phe-406 in a single α subunit is responsible for photoinactivation and that cross-linking is the result of a secondary reaction of the free 4-aminoquinaldinium moiety of dequalinium tethered to the phenylalanine side chains with side chains in the segment of the β subunit participating in the cross-links. The following considerations led to this interpretation. Although it is clear that substantial cross-linking of the derivatized phenylalanine residues to a site or sites contained within residues 440–459 of the β subunit accompanies inactivation, cross-linking does not appear to be necessary for the photoinactivation observed. This is clear from the observation that 4-amino-1-octylquinaldinium is a reversible inhibitor of MF_1 in the absence of light and causes photoinactivation when irradiated with the enzyme at 350 nm. When labeled enzyme is submitted to gel electrophoresis in the presence of TDAB under acidic conditions, the majority of the resolved radioactivity is bound to the α subunit and a cross-linked species of M_r about 116 000 which appears to be comprised of α and β subunits. Correlation of label incorporated with extent of inactivation suggests that maximal inactivation occurs on derivatization of a single site with [^{14}C]dequalinium, whereas about one-third of the label incorporated is the consequence of nonspecific derivatization. Therefore, the distribution of radioactivity in the species resolved by gel electrophoresis supports the contention that photoinactivation of MF_1 by dequalinium is caused by the mutually exclusive derivatization of the two phenylalanine residues in the α subunit. However, owing to the apparent lability of the linkage between the cross-linker and the participating side chains in the β subunit, other interpretations of the results are possible. For instance, one might argue that the segment of the β subunit found in the cross-links is the primary site of reaction with dequalinium or that the cross-linked sites found in the α and β subunits are derivatized simultaneously and that, in subsequent steps of the separation and analysis, unlabeled β subunit or β subunit fragment is released. Therefore, absolute identification of the primary site of reaction of dequalinium with MF_1 awaits synthesis of radioactive 4-amino-1-octylquinaldinium or another suitable radioactive alkylmonoquinaldinium.

The phenylalanine residues which participate in cross-linking are conserved in the α subunits of all F_1-ATPases which have been sequenced to date. This is illustrated for selected species in Table I along with the corresponding region of the β subunit of MF_1. Although the illustrated region is highly conserved within α subunits and within β subunits (not shown), there is only weak homology when the comparison is made between α and β subunits. It is interesting that the region of the β subunit illustrated contains the DELSEED segment which is labeled when MF_1 is inactivated by quinacrine mustard (Bullough et al., 1989b). The segment of the β subunit of MF_1 participating in the cross-links illustrated in Table II shows considerable homology with the same region of the β subunits of other F_1-ATPases, but shows very little homology with the corresponding segment of the α subunit of MF_1. Since the photochemistry of quinaldinium is virtually unexplored, it is not possible to comment on which side chain or side chains of the β subunit participate in cross-linking.

Irrespective of the location of the primary site of photoreaction of dequalinium with the enzyme, the cross-linking

Table I: Conservation of Sequence in the Region Corresponding to Residues 398–423 of the α Subunit of MF_1[a]

MF_1....	398	RELAAFAQFG SDLDAATQQL LSRGVR
YF_1....	404	REVAAFAQFG SDLDASTKQT LVRGQR
EF_1....	401	RELAAFSQFA SDLDDATRKQ LDHGQK
TF_1....	390	RELEAFAQFG SDLDKATQAN VARGAR
CF_1....	391	AELQAFAQFA SALDKTSQNQ LARGRR
MF_1-β..	381	YKSLQDIIAI LGMDELSEED KLTVSR

[a] Sequences were taken from the following references: MF_1, Walker et al. (1985); YF_1, Takeda et al. (1986); EF_1, Walker et al. (1985); TF_1, Ohta et al. (1988); and CF_1 (wheat), Howe et al. (1985). (*) Designates high conservation in the sequences of the α subunits shown; ( ) designates high conservation in the sequences of the α and β subunits shown.

Table II: Conservation of Sequence in the Region Corresponding to Residues 440–459 of the β Subunit of MF_1[a]

MF_1....	440	GFQQILAGEY DHLPEQAFYM
YF_1....	455	LFKAVLEGKY DNIPEHAFYM
EF_1....	426	GFKGIMEGEY DHLPEQAFYM
TF_1....	436	GFKEILEGKY DHLPEDRFRL
CF_1....	457	GFQLILSGEL DGLPEQAFYL
MF_1-α..	457	EPSKITKFEN AFLSHVISQH

[a] Sequences taken from the following references: MF_1, Walker et al. (1985); YF_1, Takeda et al. (1985); EF_1, Walker et al. (1985); TF_1, Ohta et al. (1988); and CF_1, Howe et al. (1985). (*) Designates high conservation in the sequences of the β subunits shown; ( ) designates high conservation in the sequences of the α and β subunits shown.

observed indicates that the binding site for dequalinium is at an interface comprised of residues near the caboxy termini of the α and β subunits. This is consistent with the earlier finding that quinacrine mustard, another amphipathic, cationic inactivator of the ATPase, modifies the $_{394}$DELSEED$_{401}$ segment of the β subunit (Bullough et al., 1989b). Dequalinium protects MF_1 against inactivation by quinacrine mustard, suggesting that there is a common binding site for inhibitory, amphipathic cations. It is possible that the DELSEED segment of the β subunit and the site or sites of cross-linking in the segment of β identified in this study are neighbors in the native, folded conformation of the carboxyl terminus of the β subunit.

To explain the noncompetitive, mixed, or uncompetitive inhibition of MF_1 exhibited by various amphipathic cations, we propose that these inhibitors bridge the α–β interface, thus restricting motion which is necessary for operation of the catalytic cycle. The finding that melittin and synthetic peptides bind tightly to this site (Bullough et al., 1989a) suggested that it might be the binding site for the naturally occurring inhibitor protein for MF_1 (Pullman & Monroy, 1963). However, contrary to this suggestion, unpublished work from our laboratory (F. E. Sundquist and W. S. Allison, unpublished studies, 1989) has shown that inhibitor proteins prepared from bovine heart and yeast mitochondria do not protect their respective F_1-ATPases against inactivation by quinacrine mustard. Furthermore, several inhibitory amphipathic cations including dequalinium do not affect binding of the inhibitor proteins to their respective F_1-ATPases.

Recent structural studies from Capaldi's laboratory (Gogol et al., 1990; Mendel-Hartvig & Capaldi, 1991a,b) have focused attention on the functional role of stalk proteins in the F_oF_1 complex. They suggest that these proteins relay signals between catalytic sites in F_1, which are distant from the membrane, and F_o, thus coupling transmembrane proton movement to reversible hydrolysis and synthesis of ATP. It is possible that the site in F_1 that binds inhibitory amphipathic cations has evolved to interact with one or more of the stalk proteins. It is therefore interesting that two recent studies have provided evidence that the ϵ subunit of *Escherichia coli* F_1 is at an interface of α and β subunits. Dallman et al. (1992) reported that a water-soluble carbodiimide cross-links Ser-108 of the ϵ subunit to the first glutamic acid residue in the DELSEED sequence in the β subunit. Using another approach, Aggeler et al. (1992) derivatized the cysteine residue introduced into the site-directed S108C mutant of the ϵ subunit with a tetrafluorophenyl azide–maleimide. Reconstitution of the derivatized mutant ϵ subunit with ϵ-depleted *E. coli* F_1, followed by irradiation, led to formation of an ϵ–α cross-link, thus indicating that Ser-108 is at an interface of α and β subunits. Interestingly, the yield of the ϵ–α cross-link was higher when irradiation was carried out in the presence of ATP plus Mg^{2+} than when performed in the presence of ATP plus EDTA. These observations suggest that interaction of ϵ with its binding site in α depends on the nature of the ligands bound to catalytic sites and/or noncatalytic sites. Cross-linking of ϵ to α inactivates the ATPase activity, also indicating that the site of cross-linking is in communication with the catalytic site. The intriguing observations of Aggelar et al. (1992) raise the possibility that the phenylalanine residues in the α subunit which are derivatized by dequalinium might be near the site in the α subunit which has been cross-linked to the mutant ϵ subunit of *E. coli* F_1.

Other proteins have been reported to bind dequalinium. Calcium-dependent activation of phosphodiesterase by calmodulin is inhibited by dequalinium with an $I_{0.5}$ of 1 μM (Bodden et al., 1986). The NADH dehydrogenases of the bovine heart mitochondrial and *Paracoccus denitrificans* electron transport chains are inhibited by dequalinium with $I_{0.5}$'s of 11 and 40 μM, respectively (Anderson et al., 1989). Protein kinase C is inhibited noncompetitively by dequalinium with an $I_{0.5}$ of 8–15 μM (Rotenberg et al., 1990). There are also reports that dequalinium is an antineoplastic agent that preferentially accumulates in tumor cells and thus is selectively cytotoxic, apparently by diminishing mitochondrial function (Weiss et al., 1987; Christman et al., 1990; Steichen et al., 1991). By concentrating in mitochondria, dequalinium might interfere with oxidative phosphorylation by blocking electron transport at the level of NADH dehydrogenase (Anderson et al., 1989) or by inhibiting the ATP synthase, or both.

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