Radiolabeling of Natural Adenosine Triphosphatase Inhibitor with Phenyl (^{14}C)Isothiocyanate and Study of Its Interaction with Mitochondrial Adenosine Triphosphatase. Localization of Inhibitor Binding Sites and Stoichiometry of Binding†

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ABSTRACT: The natural ATPase inhibitor (IF_1) from beef heart mitochondria was labeled with phenyl (^{14}C)isothiocyanate [(^{14}C)PITC]. Chemical labeling by (^{14}C)PITC does not modify the inhibitory properties of IF_1, provided the number of residues of (^{14}C)PITC bound per molecule of IF_1 is lower than five to six, which corresponds to the average labeling of roughly half of the available lysine residues in IF_1. This partially labeled, fully active, IF_1 was used to determine the binding stoichiometry of IF_1 with respect to F_1 and to localize the inhibitor binding sites in F_1-ATPase. The pattern of loss of ATPase activity of F_1 with increasing amounts of (^{14}C)PITC–IF_1 indicated that the ATPase activity is fully inhibited when 1 mol of IF_1 is bound to 1 mol of F_1. As F_1 contains at least 2 β subunits, this points to a half-site reactivity of F_1 with respect to IF_1. Sites of interaction between (^{14}C)PITC–IF_1 and F_1 subunits were investigated by the use of two cross-linking reagents which act as "zero length" cross-linkers, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide (EDAC) and *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline (EEDQ); the products of cross-linking were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. IF_1 was found to bind preferentially to the β subunit of F_1. Among the cross-linked products formed by reaction of EDAC or EEDQ with subunits of F_1, one of them, the $\beta\gamma$ dimer, did not accumulate when IF_1 was added to F_1 prior to cross-linking.

The natural ATPase inhibitor (IF_1),[1] discovered by Pullman & Monroy (1963) in preparations of beef heart mitochondrial ATPase, was later found in a number of H^+-linked ATPases, namely, yeast mitochondrial ATPase (Satre et al., 1975; Landry & Goffeau, 1975; Ebner & Maier, 1977), liver mitochondrial ATPase (Chan & Barbour, 1976; Cintron & Pedersen, 1979), chloroplast ATPase (Nelson et al., 1972), and bacterial ATPase (Nieuwenhuis & Bakkenist, 1977; Smith & Sternweis, 1977). It has been shown that IF_1 plays a regulatory function in oxidative phosphorylation by controlling both the reverse flow of energy from ATP to ATP-driven reactions (Asami et al., 1970; Ernster et al., 1973; Van de Stadt et al., 1973; Van de Stadt & Van Dam, 1974) and the rate of ATP synthesis (Harris et al., 1979). These data on the regulatory activity of IF_1 were drawn from experiments where interaction of IF_1 with F_1 was measured in terms of inhibition of ATPase activity. The first direct binding data obtained with a biosynthetically labeled IF_1 (Satre et al., 1975; Klein et al., 1977) were directed to the study of the binding affinity and capacity of submitochondrial particles with respect to IF_1. However, the specific radioactivity of the biosynthetically labeled IF_1 was too low to allow the accurate determination of the stoichiometry of binding of IF_1 to F_1 and to localize the inhibitor binding site(s) in F_1. These binding parameters were investigated in the present work with a chemically radiolabeled IF_1 with high specific radioactivity.

Materials and Methods

Materials. (^{14}C)PITC (10.2 mCi/mmol) and (^{3}H)NEM (161 mCi/mmol) were obtained from Amersham. Stock solutions of (^{14}C)PITC (98 mM) in Me_2SO were stored at −20 °C. (^{14}C)DCCD (52.4 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France). 1-Ethyl-3-[(dimethylamino)propyl]carbodiimide (EDAC) was purchased from Merck, *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline (EEDQ) from Aldrich, and dimethylsuberimidate (DMS) from Pierce.

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 procedure of Racker & Horstman (1967). Coupling factor F_1 was prepared by the method of Knowles & Penefsky (1972). The specific activity of purified F_1 was 70–80 μmol of ATP hydrolyzed per min per mg 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, pH 8.0, 2 mM EDTA, and 4 mM ATP. Before use, F_1 preparations were desalted by passage through a Sephadex G-50 (fine) column equilibrated with the required medium as described by Penefsky (1977). A molecular weight of 360000 for F_1 was used for calculations (Senior, 1979). IF_1 was purified by the method of Horstman & Racker (1970), as modified by Kagawa (1974) for the ethanol fractionation step. A molecular weight of 10000 for IF_1 was used for molar ratio calculations (Senior, 1979). Protein concentrations were determined by the method of Bradford (1976) using Coomassie Blue G250 for F_1 and IF_1

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[1] Abbreviations used: 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; DMS, dimethylsuberimidate; Me_2SO, dimethyl sulfoxide; EDAC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline; F_1, beef heart mitochondrial coupling factor; IF_1, beef heart ATPase protein inhibitor; 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; Cl_3AcOH, trichloroacetic acid.

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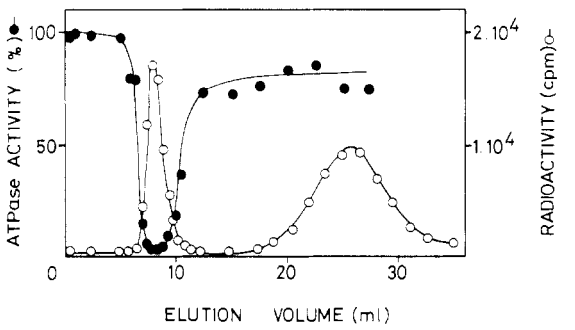

FIGURE 1: Purification of (^{14}C)PITC–IF_1. Beef heart IF_1 was labeled with (^{14}C)PITC as described under Materials and Methods. After dialysis, the (^{14}C)PITC–IF_1 preparation was loaded on a Sephadex G-25 (medium) column (1.2 × 20 cm) equilibrated with 10 mM Tris-SO_4 and 40 mM KCl, pH 7.4, and eluted with the same buffer. 1-mL fractions were collected for measurement of radioactivity (○) and inhibitor activity (●).

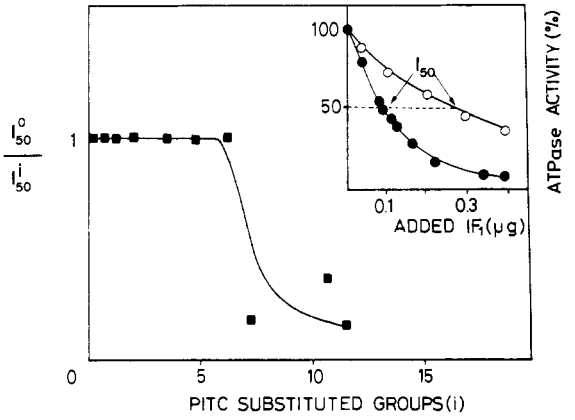

FIGURE 2: Effect of binding of PITC residues on IF_1 inhibitory activity. Beef heart IF_1 was incubated with (^{14}C)PITC under the conditions described under Materials and Methods except for time and pH. For higher incorporation of PITC, the pH of the buffer was increased to 9.0 and the incubation periods were increased to 3 h. The insert illustrates the determination of the half-inhibitory activity of unmodified IF_1 (●) and IF_1 modified with an average of 10 PITC residues bound per mol of IF_1 (○). The I_{50} values obtained for samples of IF_1 labeled with increasing concentrations of (^{14}C)PITC (i) were utilized to calculate the I_{50}^0/I_{50}^i ratio plotted on the ordinate.

and by the biuret method of Gornall et al. (1949) for AS particles. Bovine serum albumin was used as a standard.

Assay of Inhibitor Activity. The inhibitor activity of IF_1 on ATPase was measured as described by Horstman & Racker (1970) with slight modifications (Figures 1 and 2); 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 previously described (Satre et al., 1975).

Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide gels containing 0.1% $NaDodSO_4$ was carried out as described by Weber & Osborn (1969). After completion of electrophoresis, the gels were stained for 4–6 h with a solution of 0.05% Coomassie Blue R250, 25% isopropyl alcohol, and 10% acetic acid and destained according to Fairbanks et al. (1971). The densitometric traces were recorded with a Joyce-Loebl densitometer. The gels (previously fixed, stained, and destained) were frozen in solid CO_2 and cut into 1-mm slices with a Joyce-Loebl gel slicer to determine the distribution of radioactivity in the gels. The 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).

Labeling of IF_1 with (^{14}C)PITC. Beef heart IF_1 was labeled with (^{14}C)PITC by using a procedure similar to that described by Levy & Dawson (1976) for labeling immunoglobulin. (^{14}C)PITC (100 μCi, 10 mM final concentration) in Me_2SO was added to 1 mg of IF_1 in 100 mM triethanolamine buffer, pH 8.0. The final volume was 1 mL, and the final Me_2SO concentration was 10% (v/v). The mixture was incubated for 2 h under continuous stirring at room temperature. The reaction was stopped by addition of an excess of NH_2 groups (0.1 mL of 2 M Tris-HCl, pH 9.0). Excess reagent was eliminated by dialysis against 1 L of 40 mM sodium acetate and 10 mM Tris-SO_4, pH 7.4, for 3 h with two changes followed by chromatography on a Sephadex G-25 (medium) column (1.2 × 20 cm) equilibrated with the same buffer. Fractions were collected and measured for protein, radioactivity, and ATPase inhibitor activity (Figure 1). The (^{14}C)-PITC–IF_1 was eluted in the void volume, well separated from unbound (^{14}C)PITC. Fractions containing ATPase inhibitor activity were concentrated by precipitation with 10% Cl_3AcOH (w/v) (Horstman & Racker, 1970). Following the above procedure, an average of 1 mol of (^{14}C)PITC was incorporated per mol of IF_1, resulting in a specific radioactivity of 23 × 10^9 dpm/mmol. This partially labeled IF_1 was used in most of the experiments described below. It should be noted that much more (^{14}C)PITC can be incorporated into IF_1 by making the pH more alkaline.

Tryptic Peptide Mapping. (^{14}C)PITC–IF_1 (40 μg of protein, 0.84 mol of (^{14}C)PITC per mol of IF_1) was suspended in 30 mM ammonium bicarbonate, pH 7.9, and incubated at 37 °C for 3 h with TPCK–trypsin in a final volume of 0.08 mL, using a trypsin/IF_1 ratio of 1:100, and for another period of 3 h after a new addition of TPCK–trypsin to bring the trypsin/IF_1 ratio to 1:50. The reaction was stopped by 0.02 mL of 1 N acetic acid, and the digest was lyophilized. The lyophilized digest was redissolved in 5 μL of electrophoresis buffer (see below), spotted on a 10 × 10 cm cellulose thin-layer plate (F1440, Schleicher and Schüll), and submitted 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.

Binding of (^{14}C)PITC–IF_1 to F_1. F_1 (200 μg) was incubated with increasing amounts of (^{14}C)PITC–IF_1 (up to 35 μg) in 300 μL of 25 mM Mops, 0.5 mM DTT, 1 mM $MgSO_4$, and 1 mM ATP at pH 6.5 for 20 min. An aliquot fraction was assayed for ATPase activity, and in the remaining sample the IF_1–F_1 complex was separated from free IF_1 either by gel filtration or by precipitation with 50% saturated $(NH_4)_2SO_4$ (Pullman & Monroy, 1963). For gel filtration, a Sephadex G-200 (medium) column (1 × 20 cm) equilibrated with the above buffer was used. The radioactivity and the protein content of the material eluted in the excluded volume were measured. Free (^{14}C)PITC–IF_1 was also separated from (^{14}C)PITC–IF_1 bound to F_1 by a method similar to the centrifugation–filtration described by Penefsky (1977). A 1-mL disposable plastic syringe was filled with Sepharose 6B equilibrated in 0.25 M sucrose, 10 mM NaCl, 10 mM Mops, and 1 mM MgATP, pH 6.5. The column was placed into a centrifuge tube and centrifuged for 2 min in a swinging bucket IEC clinical centrifuge at low speed to pack the Sepharose. Then a 100-μL sample of the mixture of (^{14}C)PITC–IF_1 and F_1 was loaded on the Sepharose column which was centrifuged again for 2 min. Finally, 100 μL of the above medium was placed on the column and the centrifugation was repeated. The pooled eluates of the two latter centrifugations were assayed

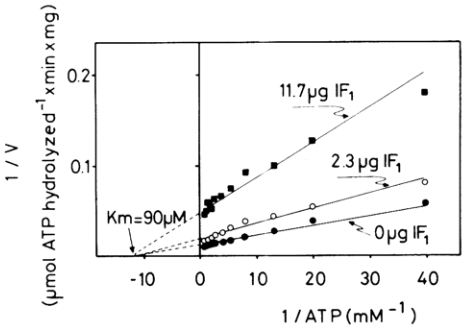

FIGURE 3: Double-reciprocal plots of ATP hydrolysis by native F_1 and F_1 inhibited by (^{14}C)PITC–IF_1. F_1 (56 μg) was preincubated for 20 min at room temperature without or with 2.3 and 11.7 μg of IF_1 in 0.25 M sucrose, 10 mM Mops, and 500 μM MgATP, pH 6.5, at a final volume of 1 mL. The ATPase activities of control enzyme (●) and inhibited enzyme (○, 2.3 μg of IF_1; ■, 11.7 μg of IF_1) were measured spectrophotometrically at 25 °C in a final volume of 2 mL containing 20 mM Tris–SO_4, pH 8.5, 0.2 mM NADH, 2 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 150 μg of pyruvate kinase, 50 μg of lactate dehydrogenase, 2 mM KCl, 10 mM sodium bicarbonate, and increasing concentrations of ATP. The velocity is given in micromoles of P_i released per minute per milligram of protein.

for ATPase activity, protein content, and radioactivity.

In the ammonium sulfate precipitation method, an equal volume of saturated ammonium sulfate was added to the solution of (^{14}C)PITC–IF_1 and F_1. After a 15-min incubation at room temperature, the precipitated protein was collected by centrifugation at maximal speed for 4 min in an Eppendorf centrifuge. The supernatant was discarded. The pellet was rinsed with 1 mL of 2 M ammonium sulfate, 0.5 mM ATP, 1 mM $MgCl_2$, 250 mM sucrose, and 10 mM Mops, pH 6.5, and then solubilized in the same medium devoid of ammonium sulfate. The protein content, the ATPase activity, and the radioactivity were measured on an aliquot sample. In control experiments, it was determined that IF_1 alone was not precipitated under these conditions.

Results

Purification and Biological Activity of (^{14}C)*PITC–*IF_1. (^{14}C)PITC was chosen to label IF_1 because of the relative abundance of lysine residues in beef heart IF_1 [11 lysine residues/mol of IF_1 as calculated from the data of Brooks & Senior (1971) and from personal data]. Another reason for the use of (^{14}C)PITC is that partial labeling by this reagent does not alter the biological activity of IF_1 (see below).

When incubation of beef heart IF_1 with (^{14}C)PITC was conducted under the conditions described under Materials and Methods, IF_1 was covalently labeled with an average of 1 mol of bound (^{14}C)PITC per mol of IF_1. A single radioactive band stained with Coomassie Blue was observed when (^{14}C)PITC–IF_1 was analyzed by NaDodSO_4–polyacrylamide gel electrophoresis. As PITC reacts with amino groups in their unprotonated form, the extent of labeling can be controlled by pH. In fact, the number of moles of (^{14}C)PITC bound per mole of IF_1 could be substantially increased by raising the pH of the reaction mixture to 9. As illustrated in Figure 2, incorporation of less than 6 mol of (^{14}C)PITC per mol of IF_1 did not alter the inhibitory efficiency of IF_1 on the ATPase activity of F_1. Raising the number of bound PITC molecules above six resulted in a abrupt decrease of the inhibitory efficiency of IF_1.

All the experiments that follow were performed with IF_1 preparations labeled with an average of 1 mol of (^{14}C)PITC per mol of IF_1. The inhibition caused by (^{14}C)PITC–IF_1 labeled in this manner was apparently noncompetitive, similar

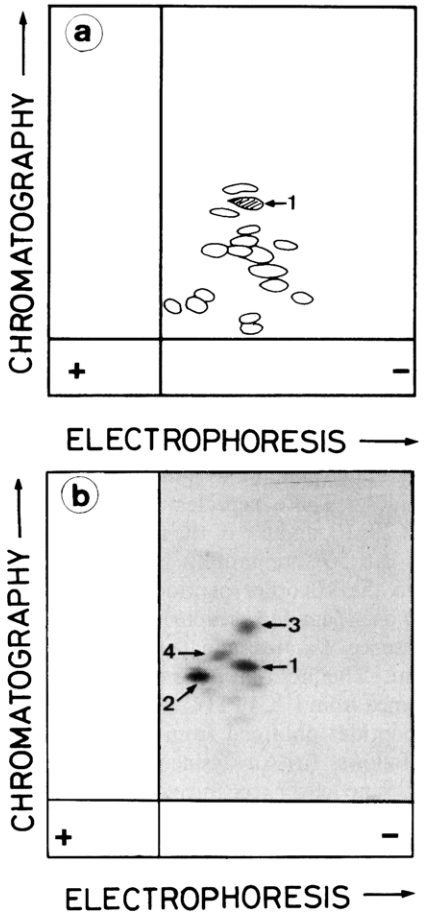

FIGURE 4: Tryptic peptide mapping of (^{14}C)PITC–IF_1. Digestion by trypsin and separation of peptides by electrophoresis followed by chromatography were as described under Materials and Methods. Peptides were visualized by ninhydrin staining and by autoradiography. A representative drawing of the ninhydrin-stained spots (a) and a photograph of the autoradiogram (b) are presented. The crosshatched circle (peptide 1) corresponds to the only radioactive ninhydrin-positive spot.

to that found for native IF_1 (Van de Stadt et al., 1973; Ernster et al., 1973), at least for a range of ATP concentrations up to 300 μM (Figure 3). However, at concentrations of ATP higher than 300 μM, plots of $1/v$ against 1/[ATP] departed from linearity probably due to tight binding of IF_1 at high concentrations of ATP and to variation in free IF_1 (Henderson, 1972). In other words, because of the high affinity of IF_1 for F_1, depending on ATP concentration, the analysis of ATPase inhibition by IF_1, based on Lineweaver–Burk plots, must be taken with caution.

Peptide Maps. A tryptic map of (^{14}C)PITC–IF_1 modified with an average of 1 PITC residue/IF_1 is presented in Figure 4. Seventeen spots were identified by ninhydrin staining (plate a). Although this number might be a slight underestimate, due to overlapping of some peptides, it agrees fairly well with the theoretical number of 17 to 18 lysine plus arginine residues found in native IF_1 (Brooks & Senior, 1971). Ninhydrin staining of the tryptic map of (^{14}C)PITC–IF_1 and unmodified IF_1 revealed the same spots with only one exception, corresponding to a supplementary radioactive spot in the case of (^{14}C)PITC–IF_1 (shadowed area, peptide 1). Tryptic digestion at different trypsin concentrations (enzyme/substrate = 1:100 or 1:50) and different periods of incubation (6–24 h) resulted in the same peptide map; furthermore, there was no material left at the origin. These data indicated that tryptic digestion of IF_1 was complete.

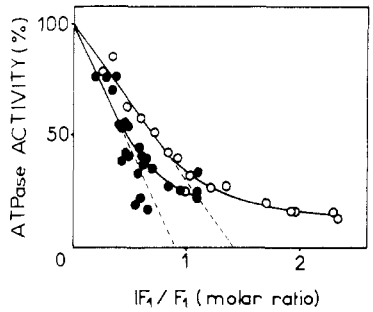

FIGURE 5: Binding of (^{14}C)PITC–IF_1 to F_1. Free and bound (^{14}C)PITC–IF_1 were separated by Sephadex gel filtration (●) or by ammonium sulfate precipitation (○) as described under Materials and Methods. Residual ATPase activity is plotted as a function of the number of moles of (^{14}C)PITC–IF_1 bound per mole of F_1.

Autoradiography (plate b of Figure 4) revealed four highly labeled peptides. These peptides, marked 1, 2, 3, and 4, contained 13, 20, 10, and 6% of the total bound radioactivity, respectively; the 50% remaining bound radioactivity was distributed in the 10 other peptides. Among the labeled peptides, only one (peptide 1) reacted with ninhydrin, probably due to the presence of a N-terminal amino acid highly sensitive to this reagent. The fact that none of the radioactive tryptic peptides obtained from (^{14}C) PITC–IF_1 (plate b) coincide with the tryptic peptides obtained from unmodified IF_1 can be explained as follows. (a) Any lysine residue which is modified by (^{14}C)PITC is no longer recognized by trypsin, and therefore trypsin digestion is more restricted with the labeled protein. (b) The new labeled peptides obtained from (^{14}C)PITC–IF_1 being larger than those arising from unmodified IF_1 migrate more slowly in electrophoresis. Being more hydrophobic due to derivatization by PITC, they migrate faster in chromatography. No free (^{14}C)PITC (characterized by a zero mobility in electrophoresis and a R_f of 1 in chromatography) was detected.

The finding that the tryptic digest of (^{14}C)PITC–IF_1 contained the same unlabeled peptides as those found in the digest of native unlabeled IF_1 plus new additional radioactive peptides suggests that, during reaction with (^{14}C)PITC, only part of the IF_1 molecules are labeled. This is consistent with the statistical distribution of radioactive IF_1 molecules calculated according to Poisson's law. Taking the case of IF_1 labeled with an average of 1 (^{14}C)PITC residue/IF_1 molecule, it can be calculated that 40% of the IF_1 molecules are unlabeled and therefore behave as native IF_1 in the tryptic map. The remaining IF_1 molecules (60%) are labeled with 1 or more than 1 (^{14}C)PITC residue per IF_1 molecule. The labeled IF_1 yielded upon trypsin digestion four highly labeled peptides (peptides 1–4) containing half of the bound radioactivity (therefore corresponding to 30% of total IF_1) and also unlabeled peptides, identical with those arising from unmodified IF_1. Therefore, each of the four major radioactive peptides should decrease the amount of unmodified peptides stained by ninhydrin by less than 10%, which explains why the tryptic peptides obtained from (^{14}C)PITC–IF_1 and from unmodified IF_1 are stained with virtually the same intensity.

Stoichiometry of (^{14}C)PITC–IF_1 Binding to Isolated F_1. After incubation of (^{14}C)PITC–IF_1 with F_1, bound (^{14}C)PITC–IF_1 was separated from free (^{14}C)PITC–IF_1 either by gel filtration or by ammonium sulfate precipitation (see Materials and Methods). A linear correlation between the binding of (^{14}C)PITC–IF_1 and the decrease of ATPase activity was obtained until 70% inhibition was attained (Figure 5). By extrapolation, one could calculate the binding stoichiometry of (^{14}C)PITC–IF_1 required for complete inhibition of ATPase activity. These stoichiometries differed, depending on whether binding was measured by gel filtration or by ammonium sulfate precipitation. They were as follows: 0.71 and 0.87 (two experiments, filtration on Sepharose 6B); 0.90 ± 0.04 (three experiments, filtration on Sephadex G-50); 0.90 ± 0.10 (three experiments, filtration on Sephadex G-200); 1.64 ± 0.13 (six experiments, ammonium sulfate precipitation). The higher value found by the ammonium sulfate precipitation technique could be due to the stabilization of low-affinity IF_1–F_1 complexes, or to a nonspecific coprecipitation of IF_1 with F_1 by ammonium sulfate, or also to the binding of an unstable oligomer form of IF_1 to F_1. This last hypothesis is supported by experimental data showing aggregation of IF_1 molecules after incubation with cross-linking reagents (see below).

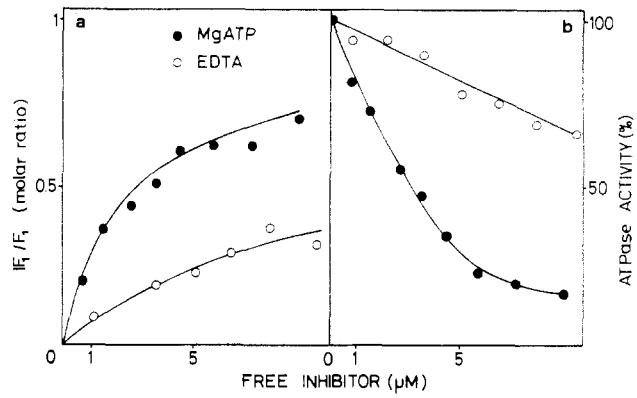

FIGURE 6: Binding of (^{14}C)PITC–IF_1 to F_1. Effect of EDTA and Mg ions. (^{14}C)PITC–IF_1 at increasing concentrations was incubated with F_1 as described under Materials and Methods in a standard incubation medium with MgATP (●) or in a medium where MgATP was replaced by 2 mM EDTA (○). At the end of the incubation, the residual ATPase activity was measured with a portion of the reaction mixture (b) and the remaining sample was filtrated on Sephadex to recover the complex made of (^{14}C)PITC–IF_1 bound to F_1 (a).

MgATP promotes the inhibition of ATPase activity in AS particles by added IF_1 (Horstman & Racker, 1970) and the binding of IF_1 to AS particles (Klein et al., 1977). In agreement with these data, (^{14}C)PITC–IF_1 was found to bind to isolated F_1 with a much higher affinity in the presence of MgATP than in the presence of EDTA; the higher binding affinity correlated to a higher efficiency of inhibition (Figure 6).

Cross-Linking of (^{14}C)PITC–IF_1 to F_1 Subunits. Purified F_1 from beef heart mitochondria contains five different subunits, α, β, γ, δ, and ϵ with molecular weights of 53000, 50000, 33000, 17500, and 7500, respectively (Senior, 1979). Previous work based on the cross-linking of F_1 subunits by the bifunctional reagent DMS along with specific labeling of the subunits had shown that the α subunits are close to each other and that the α subunit is also close to the β subunit (Satre et al., 1976; Baird & Hammes, 1977).

The cross-linking reagents used in the present work to identify which F_1 subunit binds to IF_1 were EDAC, EEDQ, and DMS. EDAC (Timkovich, 1977) and EEDQ (Belleau & Malek, 1968), two carboxyl group activating reagents, act as "zero length" cross-linkers, giving rise to amide bonds between adjacent carboxyl and amino residues at the subunit interface, whereas DMS reacts with free amino groups to form a bridge of ~12 Å.

Control assays with F_1 showed that both EDAC and EEDQ were able to generate cross-linked products (Figure 7), indicating a compact structure for F_1 with close contact between the cross-linked subunits. From the apparent molecular weights of the cross-linked products obtained with $NaDodSO_4$

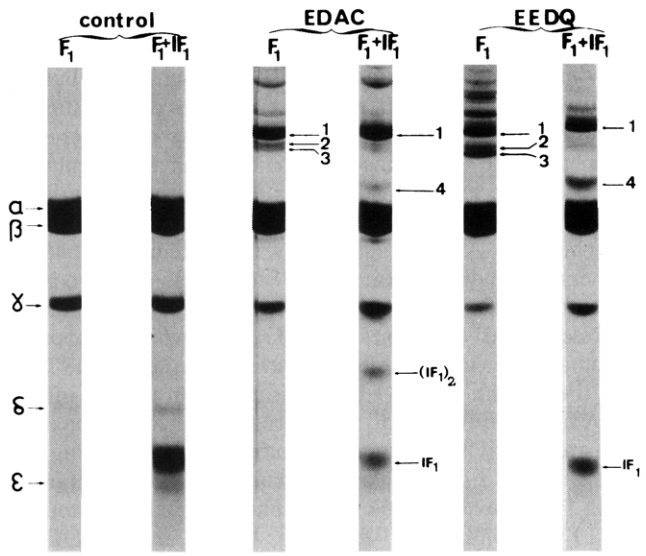

FIGURE 7: Cross-linking of F_1 and F_1–IF_1 complex with EDAC and EEDQ. F_1 (200 μg) was incubated for 15 min at 30 °C with 30 μg of (^{14}C)PITC–IF_1 in 0.25 M sucrose, 10 mM Mops, and 1 mM MgATP, pH 6.5, in a final volume of 0.2 mL. A control sample was incubated without IF_1. At this stage, ATPase activity was inhibited to ~80% by IF_1. Concentrated methanolic solutions of EDAC or EEDQ were added to give final concentrations of 5 mM EDAC or 1 mM EEDQ; the methanol concentration was less than 1%. After 20 min, cross-linking was stopped by addition of 1% (w/v) $NaDodSO_4$. After further additions of 1% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.01% (w/v) bromophenol blue (tracking dye), the samples were analyzed by polyacrylamide gel electrophoresis as described under Materials and Methods.

gel electrophoresis, band 1 (M_r 102 000 ± 3000) could be tentatively identified as a dimer of the largest subunits (either $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$) and bands 2 (M_r 90 000 ± 3000) and 3 (M_r 87 000 ± 3000) as $\alpha\gamma$ and $\beta\gamma$ dimers, respectively (Figure 7).

The cross-linking pattern of the IF_1–F_1 complex with EDAC and EEDQ compared to that of F_1 alone revealed an extra band (band 4, M_r 65 000–67 000) and the disappearance of bands 2 and 3. On the basis of the molecular weight, band 4 could be tentatively identified as α-IF_1, or as β-IF_1, or possibly as a mixture of the two dimers. A faint band with the same mobility as band 4 was observed after cross-linking with DMS, but it was less marked than the band obtained with EDAC and EEDQ, indicating that EDAC and EEDQ cross-link IF_1 and F_1 more efficiently than DMS does.

In the experiment presented in Figure 8, (^{14}C)PITC–IF_1 was cross-linked with F_1 in the presence of EEDQ. After $NaDodSO_4$–polyacrylamide gel electrophoresis, the gel was cut into slices for the determination of radioactivity. The radioactivity profile shows that (^{14}C)PITC–IF_1 is indeed associated with the new band 4 (Figure 8). Furthermore, oligomeric forms of (^{14}C)PITC–IF_1 (presumably di-, tri-, and tetramers) also accumulated, corresponding to peaks II, III, and IV, respectively. These same oligomeric forms were found when unlabeled IF_1 alone was cross-linked by EEDQ. It is noteworthy that the cross-linked products obtained by cross-linking F_1 with either unmodified IF_1 or (^{14}C)PITC–IF_1 were similar. Therefore, labeling IF_1 by PITC does not significantly disturb the F_1–IF_1 interaction and the topology of the F_1–IF_1 complex.

A better insight into the identity of band 4 was provided by specific chemical labeling of subunits α and β of F_1. The α, γ, and ϵ subunits of beef heart F_1 possess SH groups in contrast to subunit β (Senior, 1975) and to IF_1 (Brooks & Senior, 1971); subunit α and also subunits γ and ϵ can thus be labeled with (^{3}H)NEM. On the other hand, subunit β in isolated F_1 can be specifically labeled with (^{14}C)DCCD

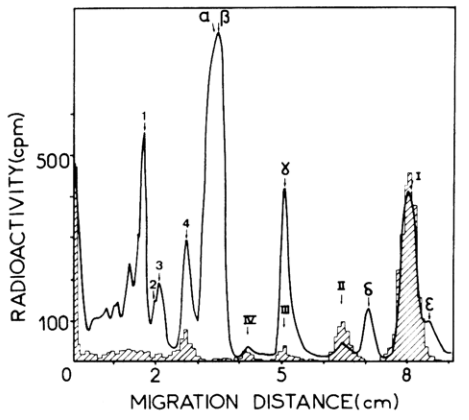

FIGURE 8: Radioactivity profile of the cross-linked (^{14}C)PITC–IF_1–F_1 complex. The complex made of (^{14}C)PITC–IF_1 and F_1 was cross-linked with 1 mM EEDQ and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described in the legend to Figure 7. The ^{14}C radioactivity profile corresponds to the shadowed area and the densitometric trace at 600 nm to the plain line. Peaks II, III, and IV are presumably di-, tri-, and tetramers of (^{14}C)PITC–IF_1, respectively.

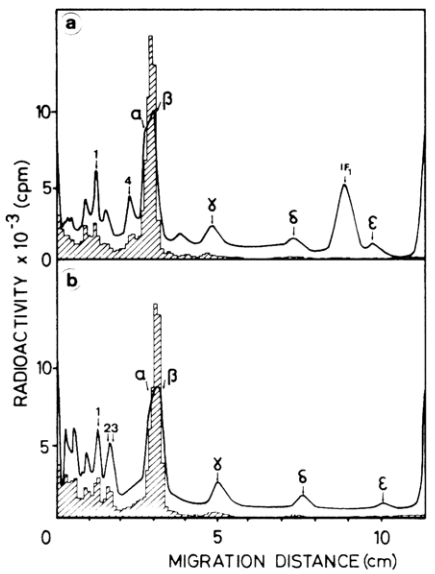

FIGURE 9: (^{14}C)DCCD labeling of cross-linked F_1 and F_1–IF_1 complex. (a) The F_1–IF_1 complex was cross-linked with 1 mM EEDQ as described in Figure 7, except that unlabeled IF_1 was used instead of (^{14}C)PITC–IF_1. (^{14}C)DCCD (100 μM) was then added and allowed to react for 0.5 h at 30 °C, which resulted in the binding of ~1.2 mol of (^{14}C)DCCD per mol of F_1 or F_1–IF_1. Free (^{14}C)DCCD was eliminated 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 (crosshatched area) is shown together with the densitometric trace at 600 nm. (b) Control cross-linking assay carried out with F_1 alone (without IF_1).

(Pougeois et al., 1979). As illustrated in Figures 9 and 10, band 4 was clearly labeled with (^{14}C)DCCD but not with (^{3}H)NEM. On the basis of the molecular weight corresponding to band 4 (65 000–67 000) and its specific labeling by (^{14}C)DCCD, the cross-linked product in band 4 can be identified as a β-IF_1 dimer. If any α-IF_1 dimer was formed, the amount was below the limit of detection. There was no evidence of a cross-linked product between IF_1 and subunit γ based on the labeling of subunit γ by (^{3}H)NEM.

Two of the cross-linked products of F_1, bands 2 and 3, were identified as $\alpha\gamma$ and $\beta\gamma$ dimers on the basis of their molecular weights and also labeling by (^{14}C)DCCD for the $\beta\gamma$ dimer (Figure 9). Binding of IF_1 to F_1 resulted in quenching of band 2 and almost complete disappearance of band 3 (Figure 7); this indicates that the binding of IF_1 to subunit β of F_1 prevents

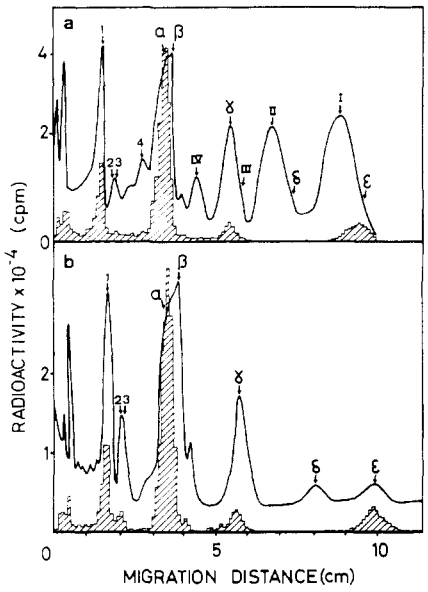

FIGURE 10: (^{3}H)NEM labeling of cross-linked F_1 and F_1–IF_1 complex. (a) The F_1–IF_1 complex was cross-linked with 5 mM EDAC as described in Figure 7, except that unlabeled IF_1 was used instead of (^{14}C)PITC–IF_1. The samples were loaded onto Sephadex G-50 (fine) columns equilibrated in 20 mM sodium phosphate, pH 7.0 (Penefsky, 1977). To the fraction corresponding to the excluded volume, 3 mM (^{3}H)NEM and 1% (w/v) $NaDodSO_4$ were added and allowed to react for 30 min at 30 °C. The samples were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The ^{3}H radioactivity profile (crosshatched) is shown together with the densitometric trace at 600 nm. (b) Control cross-linking assay carried out with F_1 alone (without IF_1).

cross-linking of subunit γ not only to subunit β but also to subunit α.

Discussion

This paper describes the chemical labeling of beef heart IF_1 by (^{14}C)PITC and the use of the radiolabeled IF_1 to investigate some binding parameters of IF_1 with respect to F_1. Only partial labeling of lysine residues in IF_1 is compatible with the expression of full inhibitory activity of IF_1. Up to 6 PITC residues/molecule of IF_1 can be introduced without noticeable loss of biological activity. The binding of more PITC residues resulted in a 80–90% inactivation of IF_1. If all lysine residues had equivalent reactivity with respect to PITC, the loss of activity upon PITC binding would be strictly proportional to the number of bound PITC molecules and the dose–effect curve would be a straight line, starting from the origin. In other words, binding of 6 PITC residues/molecule of IF_1 would result in 50% inhibition; obviously this is not the case. There is in fact a sharp threshold of inactivation of IF_1 corresponding to the binding of more than 6 PITC residues/mol of IF_1. Therefore, one must conclude that PITC binds preferentially to specific lysine residues. This is in agreement with the analysis of radioactivity in tryptic peptides of (^{14}C)PITC–IF_1 obtained with a labeling ratio of 1 (^{14}C)PITC residue/molecule of IF_1; 4 peptides out of the 14 detected by autoradiography contained 50% of the radioactivity. This again indicates the preferential reactivity of the lysine residues in these peptides to (^{14}C)PITC, probably because of a better accessibility of the reagent or a more appropriate environment.

The binding stoichiometry of (^{14}C)PITC–IF_1 to F_1 has been investigated with the (^{14}C)PITC–IF_1–F_1 complex recovered either after filtration on Sephadex or by precipitation with ammonium sulfate. Ratios close to 1 were consistently obtained in eight experiments performed by gel filtration. The ammonium sulfate precipitation technique gave slightly higher ratios, with an average of 1.6. It is probable that monomers and oligomers of IF_1 coexist in equilibrium, as revealed by cross-linking assays. Ammonium sulfate precipitation, but not gel filtration, may stabilize oligomeric forms of IF_1, thus resulting in the recovery of F_1–IF_1 complexes of higher stoichiometry. The 1:1 binding stoichiometry is in agreement with previously reported data on the interaction of IF_1 with F_1, based on the Easson and Stedman treatment of inhibition of mitochondrial ATPase activity by IF_1 (Klein et al., 1977; Gomez-Fernandez & Harris, 1978). F_1 has an oligomeric structure corresponding to either $\alpha_2\beta_2$ or $\alpha_3\beta_3$. It therefore possesses at least two copies of the β subunit which is supposed to contain the catalytic site [for review, cf. Senior (1979)]. Yet, the binding of only 1 IF_1/F_1 is sufficient to fully inhibit the ATPase activity of F_1. This result, which is typical of half-site reactivity, corroborates other data concerning the inactivation of F_1 by 4-chloro-7-nitrobenzofurazan (Ferguson et al., 1975), by butanedione and phenylglyoxal (Marcus et al., 1976; Kohlbrenner & Cross, 1978), and by DCCD (Pougeois et al., 1979).

For localization of the binding site of IF_1 on F_1, the patterns obtained upon cross-linking the F_1–IF_1 complex and F_1 alone were compared. By use of the carboxyl group activating reagents EDAC and EEDQ, two efficient zero length cross-linkers, we were able to isolate a major labeled cross-linked product consisting of (^{14}C)PITC–IF_1 and the β subunit of F_1. The β subunit in the β-IF_1 product was identified by specific labeling with (^{14}C)DCCD (Pougeois et al., 1979). (^{3}H)NEM, which labels the α, γ, and ϵ subunits but not the β subunit of F_1 (Senior, 1975), was not present in significant amounts in the cross-linked product obtained with IF_1, indicating that the α subunit does not directly interact with IF_1.

Cross-linked products obtained by reaction of EEDQ and EDAC with F_1 point to close interactions between subunits α and β, α and γ, and also β and γ. Binding of IF_1 to F_1 prevented the formation of the cross-linked product $\beta\gamma$, suggesting that IF_1 may interact with the β subunit at a site which is close to the γ subunit; accumulation of the $\alpha\gamma$ dimer was also decreased, though less markedly than that of $\beta\gamma$. It is possible that the binding of PITC–IF_1 to the β subunit of F_1 either prevents cross-linking of the β and γ subunits in F_1 by direct competition with the cross-linking reagent or induces some conformational changes that weaken interactions between the β and γ subunits and disorganize the compact structure of the three major subunits α, β, and γ in F_1.

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Proton Translocation Catalyzed by the Electrogenic ATPase in the Plasma Membrane of *Neurospora*†

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ABSTRACT: ATP hydrolysis catalyzed by the plasma membrane ATPase (ATP phosphohydrolase, EC 3.6.1.3) located on the outer surface of functionally inverted plasma membrane vesicles isolated from the eukaryotic microorganism *Neurospora crassa* gives rise to the generation of an interior positive membrane potential ($\Delta\Psi$) [Scarborough, G. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1485–1488]. The studies presented here demonstrate that the electrogenic ion in this process is H^+. In the presence of MgATP and the permeant anion SCN^-, isolated *Neurospora* plasma membrane vesicles catalyze the concentrative uptake of the ΔpH probe [^{14}C]imidazole, and this uptake is markedly inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which demonstrates MgATP-dependent intravesicular acidification (ca. 2 pH units). MgATP-dependent [^{14}C]imidazole uptake (ΔpH generation) and MgATP-dependent [^{14}C]SCN^- uptake ($\Delta\Psi$ generation) exhibit identical saturation kinetics with respect to the concentration of MgATP and are inhibited in parallel by increasing concentrations of the electrogenic ATPase inhibitors orthovanadate and diethylstilbestrol, which indicates that $\Delta\Psi$ and ΔpH are generated by the same enzyme. The fluorescent pH indicator, fluorescein-labeled dextran, placed inside the vesicles during the isolation procedure, exhibits marked time-dependent fluorescence quenching upon the addition of MgATP and SCN^-, and the fluorescence response is reversed by orthovanadate, CCCP, and nigericin plus K^+, which independently demonstrates intravesicular acidification energized by the plasma membrane ATPase. The results of these experiments provide convincing evidence that the electrogenic ATPase in the plasma membrane of *Neurospora* is a proton pump.

It has been recognized for some time that the plasma membrane of the eukaryotic microorganism *Neurospora crassa* maintains a transmembrane electrical potential ($\Delta\Psi$) of approximately 200 mV (interior negative) (Slayman, 1965). On the basis of electrophysiological studies that correlated $\Delta\Psi$ with intracellular ATP levels, Slayman et al. (1970, 1973) proposed that $\Delta\Psi$ is generated by an electrogenic ATPase located in the plasma membrane. Upon the development of the concanavalin A method for isolating *Neurospora* plasma membranes (Scarborough, 1975), it became possible to demonstrate the existence of an ATPase in the *Neurospora* plasma membrane, and the biochemical properties of this enzyme were subsequently characterized (Scarborough, 1977; Bowman &

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