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N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a New Inhibitor of the Mitochondrial F_1-ATPase†

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ABSTRACT: The ATPase activity of F_1-ATPase and AS-submitochondrial particles is progressively and irreversibly inhibited by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a specific carboxyl reagent. Reaction of 1 mol of EEDQ per active site of F_1-ATPase results in a total inactivation of the enzyme. Inactivation of soluble and membrane-bound ATPase by EEDQ is pH and temperature-dependent. pH titration shows that half-maximal inactivation is obtained at pH 7.3–7.5, suggesting that the EEDQ-reactive carboxyl group has an unusually high p*K* value. EEDQ inactivation is markedly increased at temperatures above 10 °C. Protection against EEDQ inactivation of F_1-ATPase is afforded by nucleophiles including mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine. Protection requires that these compounds are added either prior to or together with the enzyme. When added after EEDQ, the nucleophiles stop the development of the inactivation, without regeneration of the activity. Mg^{2+}, Mn^{2+}, and to a lesser extent Ca^{2+} exhibit a protective effect against EEDQ inactivation. ATP affords slight protection; ADP and AMP are without effect. Partial inhibitions caused by EEDQ and dicyclohexylcarbodiimide are additive. EEDQ does not modify the increase in fluorescence due to the formation of the aurovertin–ATPase complex, but it prevents the quenching effect of ATP on the fluorescent intensity of the aurovertin–ATPase complex. On the other hand, it has no effect on the enhancement of fluorescence of the aurovertin–ATPase complex caused by addition of ADP.

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[1], (Figure 1), a highly specific reagent for the activation and modification of carboxyl groups, was introduced by Belleau et al. (1968; also Belleau & Malek, 1968) and characterized initially as a potent depressant of the central nervous system. It has since been used successfully to probe carboxyl groups in serine hydrolases by Belleau et al. (1969) and in the ATPase of the erythrocyte membrane and found to behave in this respect like carbodiimide (Godin & Schrier, 1970). More recently the inhibitory effect of EEDQ on NADH oxidation and membrane-bound ATPase in submitochondrial particles has been briefly reported by Beechey & Cattell (1972). However, these authors did not pursue their investigation as they suspected that EEDQ lacked the required potency and specificity. In this paper, we describe the detailed characteristics of inactivation of isolated F_1-ATPase from beef heart mitochondria by EEDQ. We show that EEDQ acts at, or close to, the same site as dicyclohexylcarbodiimide on F_1-ATPase. It is in fact known that dicyclohexylcarbodiimide binds to two

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[1] Abbreviations used: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Mops, morpholinopropanesulfonic acid buffer; A particles, submitochondrial particles prepared by sonication of beef heart mitochondria in an ammonia solution at pH 9.2; AS particles, submitochondrial particles prepared by treatment of A particles with Sephadex G-50; AS particles are devoided of the natural ATPase inhibitor; F_1-ATPase, mitochondrial ATPase.

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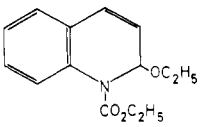

FIGURE 1: Structure of EEDQ.

regions of the mitochondrial ATPase complex, the membrane sector (Beechey et al., 1967; Roberton et al., 1968; Cattell et al., 1970) and the F_1-ATPase (Penefsky, 1967; Beechey et al., 1975). Some of our results suggest that EEDQ interferes with the binding of Mg^{2+}-ATP to the nucleotide site(s) of F_1-ATPase.

Materials and Methods

EEDQ (gold label grade) was purchased from Aldrich. It was used as a methanolic solution; appropriate controls were run with methanol alone.

Beef heart submitochondrial particles (sonic particles) depleted of their endogenous inhibitor protein (AS particles) were prepared by the procedure of Racker & Horstman (1967). Beef heart mitochondrial soluble F_1-ATPase was purified and stored at 4 °C as an ammonium sulfate suspension according to Knowles & Penefsky (1972). Its specific activity varied between 80 and 100 μmol per min per mg of protein under the described assay conditions. The enzyme used for inactivation experiments was prepared at room temperature as follows. After centrifugation to remove the ammonium sulfate, the protein pellet was suspended in the required buffer (see Results) and loaded onto a Sephadex G-50 (fine) column equilibrated with the same buffer and prepared in a 1-mL syringe as described by Penefsky (1977). The excluded fraction containing F_1-ATPase, obtained after centrifugation, was used for further assays as detailed in the text. Unless otherwise indicated, initial velocity measurements of ATPase activity were carried out at 30 °C in a total volume of 0.5 mL. The reaction mixture contained 40 mM Tris-HCl, 10 mM ATP, 5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, pH 8.0. The reaction was started by addition of the enzyme fraction and stopped after 5 min by 0.2 mL of 50% trichloroacetic acid. Inorganic phosphate released was determined by the Fiske & Subbarow (1925) method. An alternative method detailed in the legend of Figure 2 was based on the disappearance of NADH at 340 nm with the coupled enzymatic system (pyruvate kinase and lactate dehydrogenase). Aurovertin D was purified from cultures of *Calcarisporium arbusculum* (NRRL 3705) as described by Osselton et al. (1974) for aurovertin B. Aurovertin D was stored at −20 °C as an ethanolic solution protected from light. Its concentration was calculated using a molar absorption coefficient of 34 400 at 372 nm (Mulheirn et al., 1974). Aurovertin fluorescence was measured in a Perkin-Elmer MPF2A fluorimeter at 25 °C (excitation 365 nm, emission 470 nm).

The protein concentration of F_1-ATPase preparations was determined with the Folin Ciocalteu reagent (Zak & Cohen, 1961) using bovine serum albumin in the same buffer as a standard.

Results

Irreversible Inactivation of Beef Heart ATPase by EEDQ. In preliminary assays, it was found that incubation of submitochondrial AS particles or purified F_1-ATPase with EEDQ resulted in a progressive decrease of ATPase activity. The enzyme activity could not be restored after gel filtration or dilution of either the AS particles or F_1-ATPase, indicating

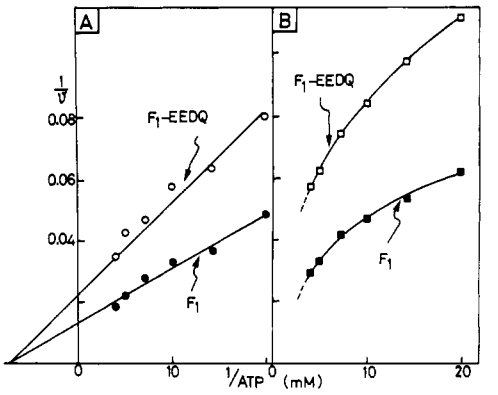

FIGURE 2: Double-reciprocal plots of ATP hydrolysis by native F_1-ATPase and F_1-ATPase partially inactivated by EEDQ. F_1-ATPase (0.50 mg/mL) was preincubated for 10 min at 24 °C with 0.4 mM EEDQ in 40 mM Mops buffer, 2 mM EDTA, final pH 7.0. Then the reaction mixture was diluted with an equal volume of 50 mM Tris-HCl, 2 mM dithiothreitol, and the pH was adjusted to 8.0. (A) The ATPase activities of control enzyme (●) and EEDQ-modified enzyme (○) were measured spectrophotometrically at 30 °C in a final volume of 2 mL containing 50 mM Tris-HCl, 240 mM sucrose, 0.2 mM NADH, 1 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 50 μg of pyruvate kinase, 25 μg of lactate dehydrogenase, 10 mM KCl, 10 mM sodium bicarbonate (final pH 8.0), and increasing concentrations of ATP. (B) The activity of untreated F_1-ATPase (■) and EEDQ-modified ATPase (□) was determined as in A except that 10 mM sodium bicarbonate was omitted. The velocity is given in μmol of inorganic phosphate released per min per mg of protein.

that EEDQ inhibition is irreversible. However, the development of EEDQ inactivation could be stopped by addition of a thiol compound (see below). In kinetic studies reported in Figures 2A and 2B, use has been made of dithiothreitol to stop EEDQ inactivation for further ATPase assay. In Figure 2A, the ATPase assay medium was supplemented with sodium bicarbonate, an activator anion which abolishes the negative cooperativity, typical of F_1-ATPase with ATP as the substrate (Ebel & Lardy, 1975). EEDQ decreased the V_{max} value of ATP hydrolysis, but not the K_M-ATP, which may be interpreted to mean that binding of EEDQ to F_1-ATPase does not modify the affinity of the enzyme for its substrate, but inactivates the enzyme in an irreversible manner. In the absence of sodium bicarbonate (Figure 2B), partially inactivated F_1-ATPase still exhibited negative cooperatively similar to the untreated F_1-ATPase. In keeping with the postulate that negative cooperativity with ATP is due to binding of ATP not only to catalytic, but also to regulatory sites (Schuster et al., 1975), it is inferred that EEDQ does not modify the regulatory site(s) of F_1-ATPase.

Time Course of Inactivation of ATPase by EEDQ. Determination of the Number of Reactive Group(s) per Active Site. EEDQ was incubated at different fixed concentrations for different periods of time with soluble F_1-ATPase or with submitochondrial particles. EEDQ concentrations were in large excess with respect to that of the enzyme. The time course of inactivation followed pseudo-first-order kinetics until 90–95% of inactivation was reached (Figure 3). The half-time of inactivation ($T_{1/2}$) varied as a function of the inhibitor concentration. The kinetic data have been plotted according to Levy et al. (1963) in order to calculate the number of inhibitor molecules able to react per active site of F_1-ATPase (Figure 4). The plot of the log of $1/T_{1/2}$ against the log of the inhibitor concentration gave a straight line with a slope equal to the number of inhibitor molecules reacting with each active site to form an inactive complex. The slope value was very close to 1, both for soluble F_1-ATPase and for submitochondrial

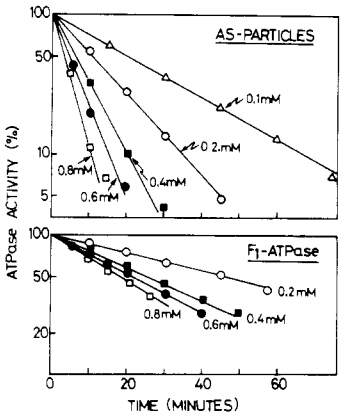

FIGURE 3: Kinetics of inactivation of ATPase activity by EEDQ. AS particles (2.8 mg/mL) or F_1-ATPase (0.25 mg/mL) were incubated at 24 °C in 40 mM Mops buffer, 2 mM EDTA, pH 7.0 (AS particles) or pH 7.5 (F_1-ATPase), with 0.1 mM (△), 0.2 mM (○), 0.4 mM (■), 0.6 mM (●), and 0.8 mM (□) EEDQ. At the indicated times, aliquot samples were diluted 20-fold in 50 mM Tris-HCl (pH 8.0) and immediately assayed for ATPase activity as described in Materials and Methods.

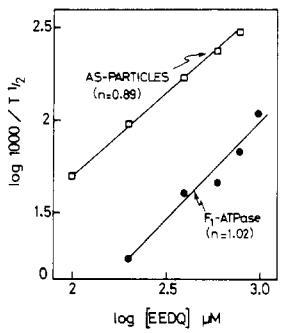

FIGURE 4: Determination of the number, n, of reactive EEDQ per active site of ATPase. The data of Figure 4 were plotted as log ($1000/T_{1/2}$) against the logarithm of EEDQ concentrations. $T_{1/2}$ is the half-time of inactivation (in min). The slope values are n = 0.89 for AS particles (□) and n = 1.02 for F_1-ATPase (●).

particles, suggesting that 1 mol of inhibitor binds to 1 mol of active site in the F_1-ATPase.

Effects of pH and Temperature on the Inactivation of Mitochondrial ATPase by EEDQ. Inactivation of soluble and membrane-bound ATPase by EEDQ was measured between pH 6.0 and pH 9.0, i.e., in a range of pH where ATPase is stable. As shown in Figure 5, sigmoidal curves of inactivation were obtained, inactivation being more marked at acidic pH. Half-maximal inactivation was found around pH 7.3–7.5. Virtually no inactivation was observed at, or above, pH 8.0.

The effect of temperature was assayed only with submitochondrial particles because of the cold sensitivity of F_1-ATPase. Inactivation of the ATPase activity by EEDQ markedly increased at temperatures above 10 °C (Figure 6).

Protection by Adenine Nucleotides and Divalent Cations. The effect of adenine nucleotides and divalent cations against EEDQ inactivation of soluble F_1-ATPase or submitochondrial particles is summarized in Table I. ATP afforded a slight protection, whereas ADP and AMP had no effect. It is of interest that the rate of inactivation was significantly decreased by the divalent cations, Mn^{2+}, Mg^{2+}, and Ca^{2+}; for example, 10 mM $MnSO_4$ or 10 mM $MgCl_2$ increased the half-time of inactivation of F_1-ATPase by a factor of 2. The protective effect of cations was independent of the accompanying anion, chloride, or sulfate.

Prevention of Inactivation by Other Reagents. A number

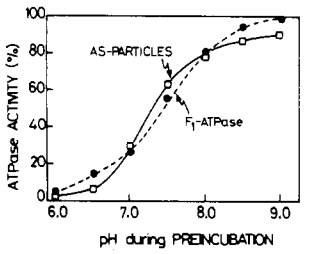

FIGURE 5: pH dependence of inactivation of ATPase by EEDQ. F_1-ATPase (0.9 mg/mL) or AS particles (2.7 mg/mL) were preincubated for 30 min at 24 °C with 0.4 mM EEDQ for F_1-ATPase (●) or 0.2 mM EEDQ for AS particles (□), in 50 mM Tris buffer and 50 mM Mops buffer, at the indicated pH. Then, aliquot samples were diluted 20-fold with 50 mM Tris-HCl, 0.5 mM NH_2OH, pH 8.0, and immediately assayed for ATPase activity as described in Materials and Methods.

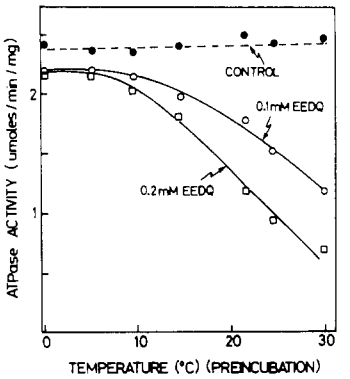

FIGURE 6: Temperature dependence of inactivation of ATPase by EEDQ. AS particles (2.7 mg/mL) were preincubated for 30 min in 40 mM Mops buffer, 2 mM EDTA, pH 7.0, without EEDQ (●) or with 0.1 mM EEDQ (○) or 0.2 mM EEDQ (□) at the indicated temperatures. Then, samples were diluted with an equal volume of 50 mM Tris buffer and 2 mM dithiothreitol. The pH was adjusted to pH 8.0 and the ATPase activity was immediately assayed at 30 °C as described in Materials and Methods.

of compounds, which have in common the property of being nucleophiles, namely 2-mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine, were found to be very effective against EEDQ inactivation of F_1-ATPase when added prior to the inhibitor (Table II). When added after the inhibitor, they could stop the course of inactivation, but there was no regeneration of the enzyme activity. These reagents per se do not stimulate ATPase activity.

The subsequent experiment was carried out to check whether 4-chloro-7-nitrobenzofurazan (Nbf-Cl) interferes with the binding of EEDQ to F_1-ATPase. At neutral pH, Nbf-Cl binds to a single tyrosine residue of the β subunit of F_1-ATPase, resulting in the inhibition of the ATPase activity. The inhibition by Nbf-Cl at, or below, pH 7.5 is fully prevented and reversed by thiols (Ferguson et al., 1974, 1975a). The EEDQ inactivation of F_1-ATPase is also prevented, but not reversed by thiols. It was therefore interesting to check whether the two inhibitors could interfere with each other. A batch of F_1-ATPase was divided into two fractions. The first fraction containing 0.15 mg of F_1-ATPase was left to react for 40 min at room temperature with 0.1 mM Nbf-Cl in 0.15 mL of 10 mM triethanolamine, 0.25 M sucrose, 2 mM EDTA, pH 7.5 in order to reach virtually full inhibition (Ferguson et al., 1975a). After reaction, the enzyme was chromatographed on a Sephadex G-50 column equilibrated with 50 mM Tris–50 mM Mops, pH 7.0, to remove excess inhibitor. The second fraction of 0.15 mg of F_1-ATPase (control) was processed under similar conditions but without Nbf-Cl. The two fractions were subdivided for further reaction with EEDQ. A portion

TABLE I: Effect of Adenine Nucleotides and Divalent Cations on Inactivation of Beef Heart Mitochondrial ATPase by EEDQ.[a]

Additions	Half-time of inact. (min)	
	F_1-ATPase	AS particles
None	15	12
ATP	20	14
ADP	14	13
AMP	15	ND[b]
$MgCl_2$	30	19
$MnSO_4$	32	23
$CaCl_2$	23	17

[a] F_1-ATPase (0.54 mg/mL) or submitochondrial particles (2.8 mg/mL) were preincubated in 50 mM Mops (pH 7.0) at 24 °C for 5 min with 10 mM nucleotides or cations, as indicated in the table. The time course of inactivation was followed after addition of 0.4 mM EEDQ and the half-time of inactivation was deduced from the semilogarithmic plot of ATPase activity as a function of time (see Figure 3). [b] ND, not determined.

TABLE II: Protective Effect of Various Reagents against Inactivation of F_1-ATPase by EEDQ.[a]

Additions	F_1-ATPase act. (%)
None	24
2 mM 2-mercaptoethanol	91
2 mM dithiothreitol	96
0.4 mM NH_2OH	84
2 mM NH_2OH	94
2 mM NH_2–NH_2	81

[a] F_1-ATPase (0.50 mg/mL) was preincubated for 5 min at 24 °C in 50 mM Mops (pH 7.0), in the presence of the reagents listed in the table. EEDQ (0.2 mM final concentration) was then added and left to react for 45 min at 24 °C. ATPase activity was assayed after a 40-fold dilution in 40 mM Tris buffer (pH 8.0).

was kept as a control, and the remaining material was incubated for 30 min at 24 °C with 0.4 mM EEDQ. At the end of the incubation period, dithiothreitol (1 mM final concentration) was added to all samples to stop EEDQ inactivation and to reverse the inhibition caused by Nbf-Cl. More than 95% of the F_1-ATPase activity was recovered in the samples containing Nbf-Cl without EEDQ. Only 30% of the activity could be recovered in the sample containing EEDQ, irrespective of whether the enzyme had been preincubated with Nbf-Cl. These data indicate that Nbf-Cl does not interfere with the binding of EEDQ and that the two compounds act at two different sites on the F_1-ATPase.

Combined Effects of EEDQ and Dicyclohexylcarbodiimide. Dicyclohexylcarbodiimide, at low concentrations, inhibits ATPase activity and oxidative phosphorylation of submitochondrial particles. In this respect, it behaves like oligomycin (Beechey et al., 1967; Roberton et al., 1968; Cattell et al., 1970). At higher concentrations and essentially at neutral or slightly acidic pH, it inhibits the hydrolytic activity of F_1-ATPase (Penefsky, 1967). As dicyclohexylcarbodiimide is also a carboxyl group-activating reagent (Kurzer & Douraghi-Zadeh, 1967), it was desirable to test whether partial inhibitory effects due to EEDQ and dicyclohexylcarbodiimide are additive. As shown in Figure 7, the percentage of inactivation measured with the mixture of the two inhibitors is equal to the percentage of inactivation calculated by summation of the two individual inactivations, according to the formula (Webb, 1963) $I_{1,2} = I_1 + I_2 - I_1 I_2$, where $I_{1,2}$ is the percentage of inhibition in the presence of both inhibitors, and I_1 and I_2, the percentages of inhibition of each separate inhibitor. These additive inhibitory effects possibly suggest that EEDQ and dicyclohexylcarbodiimide react at the same site of F_1-ATPase.

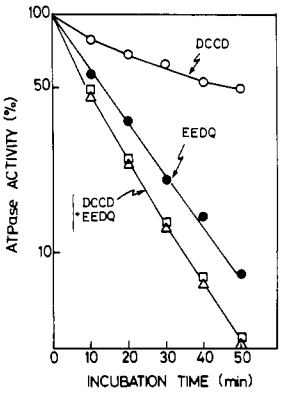

FIGURE 7: Inactivation of F_1-ATPase by DCCD and EEDQ. F_1-ATPase (0.4 mg/mL) was incubated at 24 °C in 40 mM Mops buffer, 2 mM EDTA, pH 7.0, with 0.8 mM DCCD (○) or 0.4 mM EEDQ (●) or a combination of both (□). At the indicated times, aliquot samples were diluted 20-fold and assayed for ATPase activity as described in Materials and Methods. Calculated ATPase activity in the presence of both EEDQ and DCCD (△) was derived from individual inactivation rates (○ and ●) as described in the text.

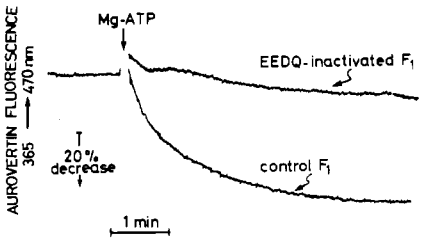

FIGURE 8: Effect of ATP on the fluorescence of the complex aurovertin-EEDQ inactivated F_1-ATPase. F_1-ATPase (1.25 mg/mL) was preincubated for 30 min with 0.4 mM EEDQ at 24 °C in 50 mM Mops, pH 7.0. At that time the enzyme was 81% inactivated. Inactivation was stopped by adding an equal volume of 50 mM Tris-HCl, 0.5 mM NH_2OH and the pH was adjusted at 8.0. The fluorescence assay was carried out at 25 °C on 0.1 mg of F_1-ATPase untreated or EEDQ inactivated in 2 mL of 0.25 M sucrose, 10 mM Tris-buffer, pH 7.4, to which 0.8 μM aurovertin (final concentration) was added. At the indicated time ATP was added (1 mM final concentration).

Effect of EEDQ on Aurovertin Fluorescence. Aurovertin binds to the mitochondrial F_1-ATPase with the formation of a fluorescent complex (Chang & Penefsky, 1973). The fluorescent intensity of the aurovertin-F_1-ATPase complex can be markedly modified upon addition of ligands. For example, it is quenched by ATP and enhanced by ADP (Chang & Penefsky, 1973). EEDQ does not prevent the increase in fluorescence due to the formation of the aurovertin-ATPase complex. However, in contrast to the control assay where the fluorescence of the aurovertin-ATPase complex is typically quenched by ATP or Mg-ATP, there is no quenching effect of ATP or Mg-ATP when the enzyme is previously treated with EEDQ (Figure 8). In contrast to ATP, the enhancement of aurovertin fluorescence caused by ADP is not altered by EEDQ (not shown).

Discussion

Some amino acid residues essential for the catalytic activity of F_1-ATPase have been recently identified by means of specific chemical reagents. Evidence for an essential tyrosine

residue was obtained in inactivation studies with Nbf-Cl (Ferguson et al., 1974, 1975a). Reversible inactivation occurs as the result of the modification of one single tyrosine residue per mole of enzyme at neutral pH; this inactivation is reversed by thiol compounds. Upon alkalinization, Nbf-Cl is transferred to a lysine residue, resulting in an irreversible inactivation (Ferguson et al., 1975b). Phenylglyoxal and 2,3-butanedione, which react selectively with the arginyl residues in proteins (Riordan et al., 1977), inactivate F_1-ATPase (Marcus et al., 1976; Frigeri et al., 1977). Dicyclohexylcarbodiimide, a carboxyl group activating reagent which has been introduced by Beechey as an inhibitor of oxidative phosphorylation, binds to the membrane sector of the ATPase complex at low concentrations (Beechey et al., 1967; Roberton et al., 1968; Cattell et al., 1970) and to the F_1 sector of the ATPase at higher concentrations (Penefsky, 1967; Beechey et al., 1975).

The present report describes some aspects of the inhibition of beef heart F_1-ATPase by EEDQ, a selective carboxyl reagent (Belleau et al., 1968; Belleau & Malek, 1968). Addition of EEDQ to membrane-bound ATPase (AS particles) or to free F_1-ATPase results in a progressive and irreversible loss of activity, suggesting a role for carboxyl group(s) in the enzyme function. Inactivation of the erythrocyte Na^+,K^+-ATPase by EEDQ has also been reported (Godin & Schrier, 1970). In contrast, the activity of phosphokinases such as adenylate kinase, hexokinase, pyruvate kinase, phosphoglycerate kinase is not altered by EEDQ (not shown). The case of phosphoglycerate kinase warrants further investigation since this enzyme possesses an essential carboxyl group capable of reacting with the Wordward's reagent K (Brake & Weber, 1974). EEDQ inhibition of the F_1-ATPase activity is accompanied by loss of the ATP binding capacity, as shown by aurovertin fluorescence measurements. Butanedione, an arginine-reactive compound, behaves like EEDQ in this respect (Marcus et al., 1976). On the contrary, Nbf-Cl does not seem to interfere with ATP binding (Ferguson et al., 1975a-c, 1976). Curiously EEDQ binding does not interfere with the enhancement of aurovertin fluorescence caused by ADP which suggests that EEDQ does not prevent the binding of ADP to F_1-ATPase. The fact that EEDQ interferes with the binding of ATP, but not with that of ADP, can be paralleled with the observation that ATP somewhat protects F_1-ATPase against EEDQ inactivation, whereas ADP has no effect. The different behavior of ATP and ADP could be explained by a different affinity of the two nucleotides for a single site in F_1-ATPase or by distinct sites for ATP and ADP. The later alternative would suggest that ATP hydrolysis and synthesis occur at different sites on F_1-ATPase (cf. Penefsky, 1974; Pedersen, 1975).

Kinetic data presented in this paper afford presumptive evidence that binding of 1 mol of EEDQ to 1 mol of active site in F_1-ATPase results in an irreversible inactivation of the enzyme. Assuming that EEDQ is a specific carboxyl reagent (Belleau et al., 1968; Belleau & Malek, 1968), it can be inferred from the kinetic data that EEDQ inactivation of F_1-ATPase is brought about by modification of one carboxyl group located at, or close to, the active site of F_1-ATPase. The study of the pH dependence of the EEDQ-inactivation of F_1-ATPase indicates that the pK of the EEDQ-reactive group is as high as 7.3-7.5. There are other examples of carboxyl groups in proteins with unusually high pK values, which are generally rationalized on the basis of a hydrophobic environment (Basch & Timasheff, 1967; Imoto et al., 1972; Braun et al., 1977; Ba Pho et al., 1977). The efficient protective effect of Mg^{2+} and Mn^{2+} against EEDQ inactivation is consistent with the binding of these cations to the EEDQ reactive carboxyl of F_1-ATPase. That a carboxyl group in the active site of F_1-ATPase acts as a ligand for divalent cations has been postulated on other grounds (Metelsky & Kozlov, 1974; Kozlov & Skulachev, 1977).

When added prior to EEDQ, the following nucleophiles, mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine, protect against EEDQ inactivation of ATPase. Godin & Schrier (1970) have reported a similar protection by nucleophiles of the erythrocyte Na^+, K^+-ATPase against inactivation by the water-soluble carbodiimide, 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide (EDAC). They suggested that EDAC-activated carboxyls might undergo nucleophilic attack by some proximal membrane nucleophile, thereby giving rise to a cross-link. The fact that nucleophiles protect against EEDQ inactivation of F_1-ATPase can be interpreted on the same basis, i.e., that the added nucleophiles compete with a nucleophile group on the F_1-ATPase, such as a sulfhydryl, a hydroxyl or an amino group, for interaction with an EEDQ-activated carboxyl. Another feasible explanation, based on the fact that EEDQ reacts with thiols (Belleau et al., 1968), is that the added nucleophile reacts with EEDQ itself, thereby decreasing the effective concentration of the inhibitor.

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Fluorine-19 Nuclear Magnetic Resonance Studies of Lipid Phase Transitions in Model and Biological Membranes†

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ABSTRACT: Fluorinated fatty acids of the general formula $CH_3(CH_2)_{13-m}CF_2(CH_2)_{m-2}COOH$ are informative spectroscopic probes of the gel to liquid-crystalline phase transitions in phospholipid dispersions and in biological membranes. We present theoretical considerations to suggest that the ^{19}F nuclear magnetic resonance line shapes are very different for frozen and fluid lipid regions. Our studies confirm this expectation for mixed phospholipid multilamellar dispersions containing a trace of difluoromyristate. The method correctly measures the onset and completion temperatures of the transition in the well-studied dimyristoylphosphatidylcholine–distearoylphosphatidylcholine system and also describes the motional behavior of the solid and fluid phases within the transition. Lipids extracted from *Escherichia coli* membranes show similar motional phenomena through the transition-temperature range according to ^{19}F nuclear magnetic resonance studies of difluoromyristate biosynthetically incorporated into the K1060B5 strain, an unsaturated fatty acid auxotroph. Intact cells or membrane vesicles show substantially different behavior from extracted lipids, indicating that membrane proteins significantly perturb the phase transition. Evidence presented in this paper also shows that the ^{19}F resonance from *Escherichia coli* phospholipids is sensitive to various intramembrane interactions. There is a general decrease in restriction of motion due to neutral lipids and an opposite effect due to the architecture of the native membrane. Neither effect is temperature sensitive. However, there are interactions in the intact membrane, affecting the ^{19}F resonance, that are temperature dependent both due to the phase-transition process and due to processes occurring at high temperatures.

Enzymatic processes within and transport across biological membranes show a temperature dependence that can be correlated to the fluidity of the phospholipid component of the membrane. For *Escherichia coli* cytoplasmic membranes, in particular, or the isolated lipids in aqueous dispersions, there are discrete changes in the slope of the temperature dependence of the specific heat, molar volume, or the intensity of a sharp X-ray diffraction line (Overath and Trauble, 1973; Overath et al., 1975; Linden et al., 1977; Jackson and Sturtevant, 1977). These changes occur at about the same temperature in both intact membranes and isolated lipids, implying that a change in the state of the lipids is being measured. Furthermore, there are changes in the temperature dependence of membrane-related enzyme activity, lactose transport or D-lactate dehy-

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