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A KINETIC AND BINDING STUDY OF THE REACTIVITY OF *ESCHERICHIA COLI* ATPase TO N-ETHOXYCARBONYL-2-ETHOXY-1,2-DIHYDRO-QUINOLINE

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SUMMARY. *Escherichia coli* H^+-ATPase (ECF_1) was inactivated in a time- and concentration-dependent manner by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a selective carboxyl group reagent. Among the subunits of ECF_1, only the β subunit was modified by EEDQ. The reaction of 1 mol of EEDQ per mol of ECF_1 resulted in total inactivation, in spite of the fact that the enzyme possesses three β subunits.

Carboxyl acid residues play a strategic function at the active center of mitochondrial, bacterial and chloroplastic H^+-ATPases (1-10). N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a selective carboxyl group reagent (11), has been shown to inactivate the mitochondrial F_1-ATPase, the reaction of 1 mol of EEDQ per active site of the enzyme resulting in total inactivation (2). The present work was carried out with purified F_1-ATPase from *Escherichia coli* (ECF_1) ; the results afford evidence for the binding of EEDQ to a carboxyl group located in the β subunit of ECF_1.

MATERIALS AND METHODS

EEDQ was purchased from Aldrich Chemical Co. Stock solutions were prepared in methanol and appropriate controls were conducted with methanol alone. [^{14}C]glycine ethyl ester (18.5 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (Saclay).

ECF_1 from *E. coli* AN180 was purified essentially as in (5) and stored at 0-4°C in 50 mM Tris-Cl, 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP, 20% (v/v) methanol, pH 7.4. Prior to inactivation assays, ECF_1 was processed by centrifugation-filtration (12) through a Sephadex G50 (fine) column equilibrated in the required buffer.

ATPase activity was determined at 37°C by a phosphate release assay (13) or by a coupled enzymatic assay measuring the disappearance of NADH in the presence of lactic dehydrogenase and pyruvate kinase/phosphoenolpyruvate as a regenerating system for ATP (2). Protein concentration was measured by a dye-binding method (14). Bovine serum albumin was used as a standard.

Abbreviations. EEDQ : N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline ; ECF_1 : the F_1-ATPase from *Escherichia coli* ; SDS : sodium dodecyl sulfate ; GEE : glycine ethyl ester ; Mops : 3-(N-morpholino) propane sulfonic acid.

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Electrophoresis was carried out using a 0.19 M Tris, 0.025 M glycine buffer, pH 8.5, containing 0.1% (w/v) sodium dodecylsulfate. The acrylamide content was 7.5% (w/v) ; the acrylamide and methylenebisacrylamide were in a ratio of 35.5 to 1 (w/w). After electrophoresis, gels were stained with Coomassie blue R250 (15) and destained in 10% (v/v) acetic acid. The distribution of radioactivity was determined as described previously (3).

RESULTS

Fig. 1 shows the double reciprocal plot of ATPase activity of control ECF_1 and EEDQ-pretreated ECF_1 as a function of increasing concentrations of the substrate Mg-ATP. Clearly, EEDQ altered the Vmax of the reaction, but not the K_M. This is an all-or-nothing inhibitory effect, typical of an inactivation process. The extent of EEDQ inactivation increased with the period of preincubation with EEDQ, according to pseudo first-order kinetics, and also with the concentration of the reagent. A plot of the log of the half-time of inactivation $T_{1/2}$, against the log of EEDQ concentration gave a straight line with a slope value close to 1. This indicates that the reaction of 1 mol EEDQ per catalytic site is sufficient to yield a fully inactive complex (Fig. 2A). The plot of $T_{1/2}$ against the reciprocal of EEDQ concentrations gave a straight line passing through the origin (Fig. 2B). This corroborates the conclusion based on the all-or-nothing inactivation data of Fig. 1 that no reversible ECF_1-EEDQ complex was formed prior to modification.

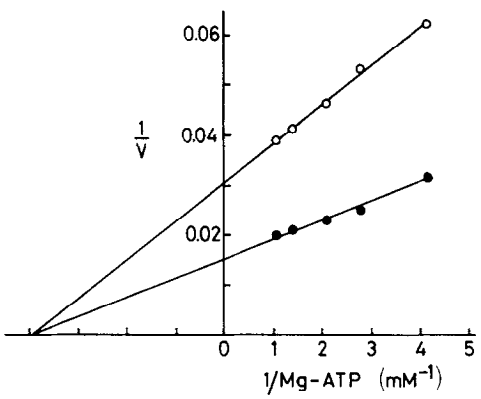


Figure 1. Double-reciprocal plots of ATP hydrolysis by ECF_1 and EEDQ-inactivated ECF_1.
ECF_1 (70 µg) was preincubated at 30°C with 50 µM EEDQ in 50 mM Mops, 0.5 mM EDTA pH 6.5. The final volume was 0.1 ml. A control was run without EEDQ under identical conditions. After 10 min, each sample was subjected to centrifugation-filtration according to Penefsky (12) on Sephadex G50 (fine) columns equilibrated in 20 mM Tris-Cl pH 7.4. The ATPase activities of control ECF_1 (•) and EEDQ-inactivated ECF_1 (o) recovered in the eluates were measured spectrophotometrically at 30°C in a final volume of 2 ml containing 50 mM Tris-SO_4, pH 8.5, 0.2 mM NADH, 4 mM phosphoenolpyruvate (cyclohexylammonium salt), 50 µg pyruvate kinase, 25 µg lactate dehydrogenase, 2 mM KCl and increasing concentrations of Mg-ATP. Velocities, V, are given in µmol of Pi released/min/mg protein.

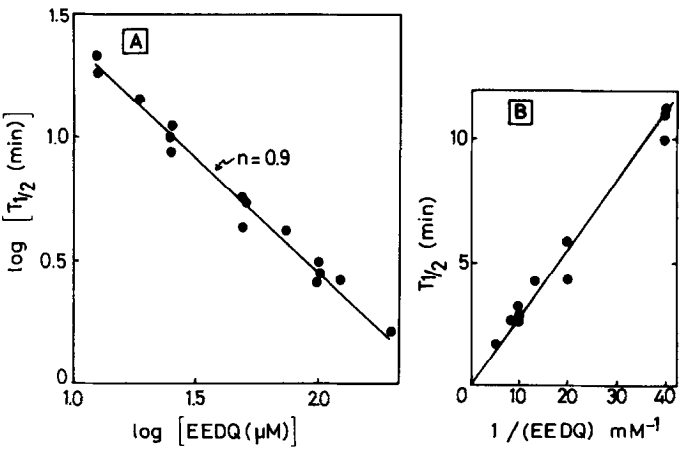


Figure 2. Kinetics of inactivation of ECF_1 by EEDQ.
A) Plot of the half times of inactivation ($T_{1/2}$) against the log of EEDQ concentration. ECF_1 (1 μg protein) was incubated at 30°C in 0.4 ml of 50 mM Mops, 0.5 mM EDTA, pH 6.5 with different concentrations of EEDQ. At various times, the samples were supplemented with 2 mM dithiothreitol and the residual ATPase activity was determined. $T_{1/2}$ was calculated from the semi-logarithmic plots of the decrease of ATPase activity as a function of time at the various concentrations of reagents.
B) Plot of $T_{1/2}$ against the reciprocal of EEDQ concentration.

Inactivation by EEDQ was pH-dependent. The rate constant for inactivation ($k_1 = 0.69/T_{1/2}$) decreased by almost an order of magnitude when the pH was increased from 6 to 8 (Fig. 3). Half-inactivation was observed near pH 7.0. The Hill plot of the data was strictly linear, and the slope value

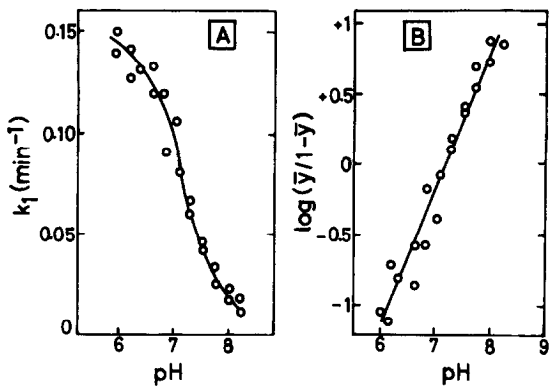


Figure 3. pH dependence of inactivation of ECF_1 by EEDQ.
ECF_1 (25 μg/ml) was incubated at 37°C in 25 mM Mops, 25 mM Tris at the indicated pHs with 100 μM EEDQ. At various times, samples were diluted 25-fold in ATPase assay medium (see "Materials and Methods") and ATPase activities were determined. Pseudo first-order rate constants (k_1) were measured from semi-logarithmic plots of ATPase activity as a function of the time of incubation with EEDQ.
A. Plot of k_1 *versus* pH
B. Hill plot of the data shown in A. $\bar{y}$ is defined as : $\bar{y} = (k_1^\circ - k_1/k_1^\circ)$, where k_1° is an estimated value at low pH ; here $k_1^\circ = 0.152\ min^{-1}$.

Table I

Effect of adenine nucleotides and divalent cations on inactivation of ECF_1 by EEDQ

Additions	$T_{1/2}$ (min)
none (5 experiments)	8 ± 2
2 mM ATP	15
5 mM ATP	18
10 mM ATP	22
10 mM ADP	9
10 mM AMP	11
10 mM $MgCl_2$	21
10 mM $CaCl_2$	26
10 mM $MnCl_2$	29

ECF_1 (0.25 mg/ml) was incubated at 37°C with 50 µM EEDQ in 50 mM Mops, 1 mM EDTA, pH 6.5, and nucleotides or divalent cations as indicated. The decrease of ATPase activity was followed as a function of time and $T_{1/2}$ were deduced from semi-log plots of ATPase activity *versus* time.

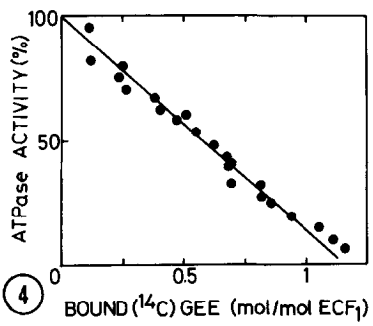
was 1. These results indicate that the carboxyl group that reacts with EEDQ has an apparent pK of 7.0 and that it binds H^+ in a non-cooperative manner.

An efficient protection was afforded by ATP and divalent cations (Table I). ATP decreased the rate of inactivation twofold when added at 2 mM and threefold when present at 10 mM ; ADP and AMP were ineffective. The cations, Mg^{2+}, Ca^{2+} and Mn^{2+}, at 10 mM, decreased the rate of inactivation by 3 to 4 times.

EEDQ-activated carboxyl groups can react with amines, such as glycine ethyl ester (GEE), to form an amide bond. By this means, and using [^{14}C]-GEE, ECF_1 could be covalently radiolabeled. The extent of covalent radiolabeling was linearly related to the extent of inactivation and no more than one mol of [^{14}C]GEE was incorporated per mol of ECF_1 (Fig. 4). To determine which subunit(s) of ECF_1 bound [^{14}C]GEE, the radiolabeled ECF_1 was subjected to SDS-polyacrylamide gel electrophoresis. Only the β subunit was found to incorporate radioactivity covalently (Fig. 5).

DISCUSSION

The use of EEDQ for the selective activation of carboxyl groups in non-aqueous media was first described by Belleau and Malek (11), and later extended to the activation of carboxyl groups of proteins in water solutions (16-18). In a previous work, it was shown that EEDQ inactivates the mitochondrial F_1-ATPase and that reaction of 1 mol of EEDQ per active site of



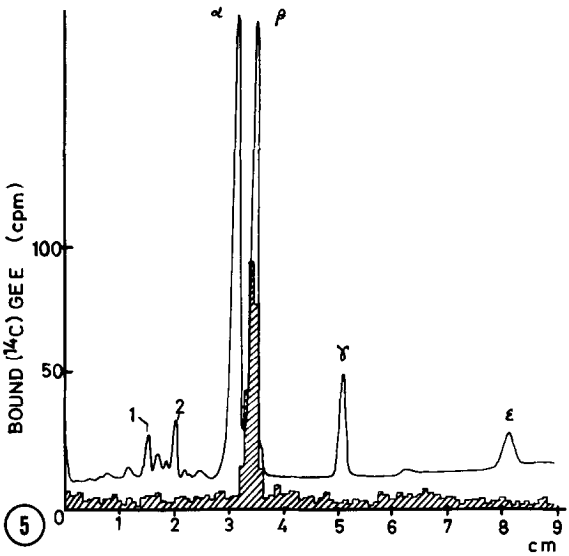


Figure 4. Correlation between inactivation of ECF_1 with EEDQ and incorporation of [^{14}C]GEE.
ECF_1 (0.36 mg/ml) was incubated with 120 µM EEDQ at 30°C in 50 mM Mops, 0.2 mM EDTA, 1.1 mM [^{14}C]GEE, pH 7.0. Aliquots were removed at intervals and subjected to centrifugation-filtration (12), prior to measurements of bound [^{14}C]GEE and ATPase activity. Control assays were carried out under identical conditions with [^{14}C]GEE, but without EEDQ. The control values for ATPase activities were 70 µmol ATP hydrolyzed/min/mg protein.

Figure 5. ECF_1 was inactivated with EEDQ in the presence of [^{14}C]GEE as described in the legend of Fig. 4 up to 68% inactivation. After removal of unbound [^{14}C]GEE by centrifugation-filtration (12), ECF_1 (8 µg protein) was subjected to SDS-polyacrylamide gel electrophoresis. The cross-linked products, referred to by numbers 1 and 2, have approximate Mr of 110,000 and 87,000.

the enzyme resulted in total inactivation (2). As shown here, ECF_1 behaves similarly to the mitochondrial ATPase with respect to inactivation by EEDQ. In particular, in both cases, EEDQ reacts with a carboxyl group of unusually high pK (pK $\simeq$ 7). High pK value for carboxyl groups in proteins are generally related to their hydrophobic environment. That this carboxyl group is involved in the binding of ATP and divalent cations is suggested by the protective effect afforded by these ligands. Most interesting is the effect of divalent cations, since the EEDQ reactive carboxyl group in ECF_1 probably binds Mg^{2+} at the catalytic site (19).

EEDQ has also been used as a cross-linking reagent (20). One may therefore wonder whether the EEDQ-mediated inactivation of ECF_1 could not be due to cross-linking of subunits. This was apparently not the case since inter-subunits cross-linking proceeded much more slowly than inactivation. In fact, accumulation of cross-linked products became detectable by SDS-gel electrophoresis only when inactivation had reached 60 to 70%.

The fact that EEDQ binds to the β subunit of ECF_1 strengthens the idea that the catalytic site of ECF_1 is located on the β subunit. As shown by the

covalent incorporation of the radioactive nucleophile [^{14}C]GEE that reacted with the EEDQ-activated carboxyl group, full inactivation coincides with the reaction of 1 mol EEDQ per mol ECF_1, in spite of the fact that there are three β subunits per ECF_1 (21). This is another example of the partial site reactivity of ECF_1, which can be considered strongly indicative of subunit-subunit interactions in ECF_1 (22).

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