EEDQ probably reacts with the Mg^{2+}-ATP catalytic sites of mitochondrial and bacterial F_1-ATPases

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The carboxyl reagent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) inactivated ATPase activities of isolated MF_1 and BF_1 when assayed in an $MgCl_2$ medium, but not in an EDTA medium. However, another carboxyl reagent, *N,N'*-dicyclohexylcarbodiimide (DCCD) was found to inhibit MF_1 and BF_1 when assayed either in the presence of $MgCl_2$ or EDTA. These data suggest that EEDQ interferes with the binding of Mg^{2+} at catalytic sites of both MF_1 and BF_1 and that EEDQ on one hand, and DCCD on the other, react with different carboxyl groups on MF_1 and BF_1.

Mitochondrial ATPase *Bacterial ATPase* *Carboxyl group* *Catalytic site*
N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline *N,N'-Dicyclohexylcarbodiimide*

1. INTRODUCTION

It was shown that isolated mitochondrial ATPase (MF_1) and *Escherichia coli* ATPase (BF_1) are inactivated by two carboxyl group reagents, namely *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and *N,N'*-dicyclohexylcarbodiimide (DCCD). In all cases, divalent cations protected the enzyme against inactivation [1–5], which is consistent with the proposal [6] that a carboxyl group in the catalytic site of F_1-ATPase acts as a ligand for divalent cations. Although ATP hydrolysis by MF_1 (and BF_1) is routinely assayed in the presence of Mg^{2+}, a low but measurable ATPase activity still occurs when MF_1 and BF_1 are incubated in an EDTA-supplemented medium. It was thought therefore that ATP hydrolysis in the presence of EDTA was a very simple method to check whether the above carboxyl chemical modifiers were acting specifically at the Mg^{2+} catalytic binding sites on MF_1 or BF_1. The results of these experiments are reported here.

Abbreviations: MF_1, beef heart mitochondrial ATPase; BF_1, bacterial ATPase; TNP-ATP, 2',3'-*O*-(2,4,6-trinitrophenyl)-ATP; CMCD, 1-cyclohexyl-3-(2-morpholino-4-ethyl)-carbodiimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; MOPS, 3-(*N*-morpholino) propane sulfonic acid

2. MATERIALS AND METHODS

TNP-ATP was synthesized according to [7]. Picrylsulfonic acid was purchased from Aldrich. TNP[γ-^{32}P]ATP was synthesized and purified using the procedure in [8]. The concentrations of the TNP-nucleotides were measured spectrophotometrically at pH 8.0, assuming a molar extinction coefficient of 26400 $M^{-1}.cm^{-1}$ at 408 nm and 18500 $M^{-1}.cm^{-1}$ at 470 nm [7,8].

Efrapeptin was provided by Dr Hamill of Eli Lilly Co. It was added at a concentration corresponding to a mass ratio of efrapeptin to MF_1 equal to 1/50; i.e., a molar ratio of efrapeptin to MF_1 of about 20/1.

MF_1 and BF_1 were prepared as in [9] and [5], respectively. The assay medium for Mg^{2+}-ATP hydrolysis contained, in a final volume of 0.5 ml, 40 mM Tris–HCl, 10 mM ATP, 5 mM $MgCl_2$,

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20 μg pyruvate kinase and 2 mM phosphoenol pyruvate, final pH 8.0 ($MgCl_2$ medium). The reaction was carried out at 37°C; it was started by addition of 3–5 μg ATPase and stopped after 2 min by addition of 0.2 ml ice-cold trichloroacetic acid (50%, w/v). The P_i released was determined as in [10]. In the case of EDTA–ATP hydrolysis the incubation medium contained 40 mM Tris–HCl, 1 mM EDTA, 10 mM ATP, final pH 8.0, final vol. 0.5 ml (EDTA medium); the temperature was 37°C. The reaction was started by addition of 200–300 μg ATPase and stopped after 3 h as above. The released P_i was determined in the supernatant after centrifugation for 5 min, at 10000 × g.

TNP–[γ-^{32}P]ATP hydrolysis was carried out in a medium consisting of 40 mM Tris–HCl, 20 μM TNP–[γ-^{32}P]ATP, and either 10 mM $MgCl_2$ or 1 mM EDTA, final pH 8.0, final vol. 0.5 ml, temp. 37°C. In the case of the $MgCl_2$ medium, the reaction was started by addition of 10 μg MF_1 or BF_1 and stopped as above after 30 s. In the case of the EDTA medium the incubation time was 15 h, and 50–70 μg MF_1 or BF_1 were used. In both cases, the $^{32}P_i$ released was determined as in [8].

EEDQ modification was performed at 20°C in 50 mM MOPS, 1 mM EDTA (pH 6.5) in the presence of 0.4 mM EEDQ. After 10 min, ATPase was equilibrated with 40 mM Tris–HCl, 1 mM EDTA (pH 8.0) using the centrifuge column method [11]. DCCD and CMCD modifications were performed in 50 mM MOPS, 1 mM EDTA, 2 mM ATP (pH 6.5) in the presence of 200 μM DCCD or 10 mM CMCD. After 40 min, the samples were treated to remove the non-reacted DCCD and CMCD as described above for EEDQ modification.

3. RESULTS

In the presence of EDTA, ATP hydrolysis by MF_1 was catalyzed at a rate 10000-fold lower than that obtained in the presence of Mg^{2+} (6 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$; i.e., 130 mol $P_i \cdot h^{-1} \cdot (mol\ F_1)^{-1}$). Hydrolysis of ATP in the presence of EDTA was not due to a contaminant since efrapeptin inhibited the reaction. Because of its high reactivity and specificity, efrapeptin (an antibiotic) is considered as one of the most reliable, non-covalent inhibitors of MF_1 [12] and, because of this, it is used to discriminate the presence of contaminant enzymes with ATPase activity [8]. The working hypothesis was that CMCD, EEDQ or DCCD might specifically modify the Mg^{2+} binding site at the catalytic sites, thus leaving the ATP hydrolysis in the presence of EDTA unaltered. MF_1 was therefore modified by the above reagents. After incubation, the reagents were removed by centrifugation–elution [11], and the activities of MF_1 were measured in the $MgCl_2$ or EDTA medium. The EEDQ-modified MF_1 was 100% active in the EDTA medium in contrast to the DCCD- and CMCD-modified MF_1 that largely lost their activity (table 1). In the $MgCl_2$ medium, MF_1 modified by EEDQ, DCCD and CMCD was in any case severely inhibited. The peptide inhibitor efrapeptin was equally effective in the $MgCl_2$ or EDTA medium.

MF_1 and BF_1 first modified by EEDQ were still able to bind [^{14}C]DCCD [2,5]. This could be due to the fact that the carboxyl group modified by

Table 1

Effect of DCCD, CMCD and EEDQ on the rate of ATP hydrolysis by MF_1 and BF_1 in $MgCl_2$- and EDTA-supplemented media (% control)

ATPase preparation	Modifier used	Rate of ATP hydrolysis	
		$MgCl_2$ medium	EDTA medium
MF_1	None	100	100
	DCCD	6	30
	CMCD	13	28
	EEDQ	30	98
BF_1	None	100	100
	DCCD	4	42
	EEDQ	25	95

Mg^{2+}-ATPase and EDTA–ATPase activities were measured as in section 2. Chemical modifications were performed as in section 2. The rates of ATP hydrolysis by MF_1 in the $MgCl_2$ and EDTA media were 73 μmol P_i released $\cdot min^{-1} \cdot mg^{-1}$ and 6 nmol P_i released $\cdot min^{-1} \cdot mg^{-1}$, respectively. For BF_1 the rates were 48 μmol P_i released $\cdot min^{-1} \cdot mg^{-1}$ and 10 nmol P_i released $\cdot min^{-1} \cdot mg^{-1}$, respectively. These values represent 100% activity. The activity of MF_1 was decreased to 15–17% of the control value by addition of efrapeptin either in the $MgCl_2$ medium or in the EDTA medium (section 2)

Table 2

Effect of EEDQ on the rate of TNP–ATP hydrolysis in $MgCl_2$- and EDTA-supplemented media (% control)

ATPase preparation	Modifiers used	Rate of TNP–ATP hydrolysis	
		$MgCl_2$ medium	EDTA medium
MF_1	None	100	100
	EEDQ	28	102
BF_1	None	100	100
	EEDQ	35	98

TNP–ATPase activities of MF_1 and BF_1 were measured as in section 2. The rates of TNP–ATP hydrolysis in the $MgCl_2$ medium were 450 nmol P_i released.min^{-1}.mg MF_1^{-1} and 350 nmol P_i released.min^{-1}.mg BF_1^{-1}, respectively. In the EDTA medium the rates were 0.10 nmol P_i released.min^{-1}.mg MF_1^{-1} and 0.18 nmol P_i released.min^{-1}.mg BF_1^{-1}, respectively

EEDQ was not the same as that modified by DCCD. This is corroborated here by the finding that, in the EDTA medium, MF_1 modified by DCCD displayed a significant loss of activity while MF_1 modified by EEDQ showed virtually no loss of activity (table 1); similar results were obtained with BF_1.

It was reported in [8] that MF_1 contains two high affinity binding sites for TNP–ATP, and that these sites are catalytic. In the presence of Mg^{2+}, TNP–ATP was hydrolyzed by MF_1 640-times more slowly than ATP; further, treatment of MF_1 by DCCD or EEDQ inactivated both the hydrolysis of ATP and TNP–ATP in the presence of $MgCl_2$ [8]. These results were confirmed, and in addition it was shown that native MF_1 and MF_1 modified by EEDQ were both fully active when ATPase activity was measured in EDTA medium with TNP–ATP as substrate (table 2); BF_1 hydrolyzed TNP–ATP at similar rates as MF_1 did. Furthermore as with MF_1, the EEDQ-modified BF_1 was inactivated when tested in the $MgCl_2$ medium, and fully active when tested in the EDTA medium.

4. DISCUSSION

The central finding in this study is that EEDQ, a highly specific reagent of carboxyl groups [13,14], inactivates MF_1 and BF_1 when the ATPase activity is tested in a $MgCl_2$ medium [1,4], but is ineffective when the ATPase activity is tested in an EDTA medium. Thus the EEDQ-reactive carboxyl group probably functions to bind Mg^{2+} at the catalytic sites of MF_1 or BF_1.

The presence of two catalytic sites on MF_1 has been demonstrated using TNP–ATP [8]; the first binding site binds TNP–ATP with an affinity too high to be measured, and the K_d value for the second binding site was 20 nM either in the absence or in the presence of Mg^{2+} [8]. Since native MF_1 and EEDQ-modified MF_1 are equally active in an EDTA medium when tested with TNP–ATP as substrate (table 2), this again lends support to the view that the EEDQ-modified carboxyl group is implicated in Mg^{2+} binding at the catalytic sites.

A last comment concerns the hydrolysis of ATP by MF_1 and BF_1 in the absence of $MgCl_2$ and in the presence of EDTA. Although ATP hydrolysis in an EDTA medium is very slow, it is measurable; when [^{14}C]ATP is used, it leads to the formation of [^{14}C]ADP after a few minutes of incubation. In other words, results of [^{14}C]ATP-binding experiments must be interpreted with caution, since the equilibration period with the enzyme lasts for a few minutes and that both [^{14}C]ADP and [^{14}C]ATP are then present in the medium.

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