

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
<i>N</i> -Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline		<i>N,N'</i> -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.

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 $26400\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 408 nm and $18500\text{ M}^{-1}\cdot\text{cm}^{-1}$ 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 $MgCl_2$,

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

20 μ g pyruvate kinase and 2 mM phosphoenol pyruvate, final pH 8.0 (MgCl₂ medium). The reaction was carried out at 37°C; it was started by addition of 3–5 μ g 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 μ g ATPase and stopped after 3 h as above. The released P_i was determined in the supernatant after centrifugation for 5 min, at 10000 \times g.

TNP–[γ -³²P]ATP hydrolysis was carried out in a medium consisting of 40 mM Tris–HCl, 20 μ M TNP–[γ -³²P]ATP, and either 10 mM MgCl₂ or 1 mM EDTA, final pH 8.0, final vol. 0.5 ml, temp. 37°C. In the case of the MgCl₂ medium, the reaction was started by addition of 10 μ g MF₁ or BF₁ and stopped as above after 30 s. In the case of the EDTA medium the incubation time was 15 h, and 50–70 μ g MF₁ or BF₁ were used. In both cases, the ³²P_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 μ M 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 MF₁ was catalyzed at a rate 10000-fold lower than that obtained in the presence of Mg²⁺ (6 nmol P_i · min^{−1} · mg^{−1}; i.e., 130 mol P_i · h^{−1} · (mol F₁)^{−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 MF₁ [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 Mg²⁺ binding site at the catalytic sites, thus leaving the ATP hydrolysis in the presence of EDTA unaltered. MF₁ was therefore modified by the above reagents. After incubation, the reagents were removed by centrifugation–elution [11], and the activities of MF₁ were measured in the MgCl₂ or EDTA medium. The EEDQ-modified MF₁ was 100% active in the EDTA medium in contrast to the DCCD- and CMCD-modified MF₁ that largely lost their activity (table 1). In the MgCl₂ medium, MF₁ modified by EEDQ, DCCD and CMCD was in any case severely inhibited. The peptide inhibitor efrapeptin was equally effective in the MgCl₂ or EDTA medium.

MF₁ and BF₁ first modified by EEDQ were still able to bind [¹⁴C]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 MF₁ and BF₁ in MgCl₂- 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

Mg²⁺-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 MF₁ in the MgCl₂ and EDTA media were 73 μ mol P_i released · min^{−1} · mg^{−1} and 6 nmol P_i released · min^{−1} · mg^{−1}, respectively. For BF₁ the rates were 48 μ mol P_i released · min^{−1} · mg^{−1} and 10 nmol P_i released · min^{−1} · mg^{−1}, respectively. These values represent 100% activity. The activity of MF₁ was decreased to 15–17% of the control value by addition of efrapeptin either in the MgCl₂ medium or in the EDTA medium (section 2)

Table 2

Effect of EEDQ on the rate of TNP-ATP hydrolysis in MgCl₂- 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 MF₁ and BF₁ were measured as in section 2. The rates of TNP-ATP hydrolysis in the MgCl₂ medium were 450 nmol P_i released.min⁻¹.mg MF₁⁻¹ and 350 nmol P_i released.min⁻¹.mg BF₁⁻¹, respectively. In the EDTA medium the rates were 0.10 nmol P_i released.min⁻¹.mg MF₁⁻¹ and 0.18 nmol P_i released.min⁻¹.mg BF₁⁻¹, respectively

EEDQ was not the same as that modified by DCCD. This is corroborated here by the finding that, in the EDTA medium, MF₁ modified by DCCD displayed a significant loss of activity while MF₁ modified by EEDQ showed virtually no loss of activity (table 1); similar results were obtained with BF₁.

It was reported in [8] that MF₁ contains two high affinity binding sites for TNP-ATP, and that these sites are catalytic. In the presence of Mg²⁺, TNP-ATP was hydrolyzed by MF₁ 640-times more slowly than ATP; further, treatment of MF₁ by DCCD or EEDQ inactivated both the hydrolysis of ATP and TNP-ATP in the presence of MgCl₂ [8]. These results were confirmed, and in addition it was shown that native MF₁ and MF₁ modified by EEDQ were both fully active when ATPase activity was measured in EDTA medium with TNP-ATP as substrate (table 2); BF₁ hydrolyzed TNP-ATP at similar rates as MF₁ did. Furthermore as with MF₁, the EEDQ-modified BF₁ was inactivated when tested in the MgCl₂ 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 MF₁ and BF₁ when the ATPase activity is tested in a MgCl₂ 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 Mg²⁺ at the catalytic sites of MF₁ or BF₁.

The presence of two catalytic sites on MF₁ 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 Mg²⁺ [8]. Since native MF₁ and EEDQ-modified MF₁ 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 Mg²⁺ binding at the catalytic sites.

A last comment concerns the hydrolysis of ATP by MF₁ and BF₁ in the absence of MgCl₂ and in the presence of EDTA. Although ATP hydrolysis in an EDTA medium is very slow, it is measurable; when [¹⁴C]ATP is used, it leads to the formation of [¹⁴C]ADP after a few minutes of incubation. In other words, results of [¹⁴C]ATP-binding experiments must be interpreted with caution, since the equilibration period with the enzyme lasts for a few minutes and that both [¹⁴C]ADP and [¹⁴C]ATP are then present in the medium.

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REFERENCES

- [1] Pougeois, R., Satre, M. and Vignais, P.V. (1978) *Biochemistry* 17, 3018-3023.
- [2] Pougeois, R., Satre, M. and Vignais, P.V. (1979) *Biochemistry* 18, 1408-1413.
- [3] Pougeois, R., Satre, M. and Vignais, P.V. (1980) *FEBS Lett.* 117, 344-348.
- [4] Satre, M., Pougeois, R., Lunardi, J., Dianoux, A.-C., Klein, G., Bof, M. and Vignais, P.V. (1978) in: *Frontiers of Biological Energetics: Electrons to Tissues* (Dutton, P.L. et al. eds) vol.1, pp.467-476, Academic Press, London, New York.
- [5] Satre, M., Lunardi, J., Pougeois, R. and Vignais, P.V. (1979) *Biochemistry* 18, 3134-3140.

- [6] Kozlov, I.A. and Skulachev, V.P. (1977) *Biochim. Biophys. Acta* 463, 23–89.
- [7] Hiratsuka, T. and Uchida, K. (1973) *Biochim. Biophys. Acta* 320, 635–647.
- [8] Grubmeyer, C. and Penefsky, H.S. (1981) *J. Biol. Chem.* 256, 3718–3727.
- [9] Knowles, A.F. and Penefsky, H.S. (1972) *J. Biol. Chem.* 247, 6617–6623.
- [10] Fiske, C.H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–400.
- [11] Penefsky, H.S. (1977) *J. Biol. Chem.* 252, 2891–2899.
- [12] Cross, R.L. and Kohlbrenner, W.E. (1978) *J. Biol. Chem.* 253, 4865–4873.
- [13] Belleau, B., Martel, R., Lacasse, G., Menard, M., Weinberg, N.L. and Perron, Y.G. (1968) *J. Am. Chem. Soc.* 90, 823–824.
- [14] Belleau, B. and Malek, G. (1968) *J. Am. Chem. Soc.* 90, 1651–1652.