Three catalytic sites in mitochondrial ATPase

E. Cerdán, M.L. Campo*, N. López-Moratalla and E. Santiago+

Department of Biochemistry, University of Navarra, Pamplona, Spain

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Kinetic data obtained after determining the hydrolytic activity of ATPase from rat liver in preparations where the enzyme had been purified, or in mitochondria, strongly suggest the existence of three different catalytic sites with different affinity for the substrate. The results obtained when measuring the ATPase activity at different substrate concentrations, and in the presence of the inhibitors KOCN or KSCN, or of the activators dinitrophenol and bicarbonate, show that the binding of these compunds to a regulatory site or sites affects in a different degree the hydrolytic activity of each catalytic site.

Mitochondrial ATPase *F_1-ATPase* *Catalytic site* *(Rat liver)*

1. INTRODUCTION

Kinetics studies of the hydrolytic activity of mitochondrial ATPase [1,2] suggest the existence of more than one catalytic site in the enzyme; their results are consistent with the presence of at least two sites with different kinetic parameters. The existence of three catalytic sites had also been suggested in [3] after the observation that the hydrolytic activity of ATPase exhibited a maximum at 3 different pH-values depending on the substrate concentration used. In [4,5], using different analogs of ATP, results consistent with the existence of 3 catalytic sites and associated with each one of the subunits of ATPase, were obtained. Two catalytic sites in beef heart mitochondrial ATPase were reported in [6], but an additional site was not ruled out.

Using rat liver mitochondrial of F_1-ATPase within a wide range of ATP–Mg^{2+} concentrations, and in the presence or absence of a variety of activators or inhibitors, the kinetic data now obtained indicate that 3 catalytic sites with different affinity for the substrate are present in the enzyme.

+ To whom correspondence should be addressed

* Present address: Department of Biological Sciences SUNY, Albany, NY, USA

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated as in [7] in 250 mM sucrose. F_1-ATPase was prepared from rat liver mitochondria as in [8].

ATPase was assayed by measuring the release of P_i essentially as in [9] in the absence of an ATP generating system. Aliquots of the F_1-ATPase of mitochondria were preincubated for 5 min at 30°C in 0.8 ml of a medium containing 50 μm Tris–acetate, at pH 7.4. The reaction was initiated by the addition of substrate (ATP-Mg^{2+}) at the appropriate concentration. The incubation was continued for 3 min and stopped by the addition of 0.1 ml 50% trichloroacetic acid. P_i was determined according to [10]. Reagent and enzyme blanks were determined in each experiment.

The K_a was determined as follows: using a constant concentration of the substrate ATP-Mg^{2+}, plots of $1/(v - v_0)$ 'versus' 1/[activator] were constructed (where v = velocity in the presence and v_0 = velocity the abscence of activator); the y-intercept represents $1/V_{max} - v_0$); slope/intercept was defined as the K_a for the activator [1]. The different K_i values were calculated as in [11].

Protein was determined as in [12]. Crystalline bovine serum albumin was used as standard.

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3. RESULTS AND DISCUSSION

3.1. *ATPase hydrolytic activity and substrate concentration*

ATPase hydrolytic activity of the isolated enzyme and that bound to the mitochondria membrane was determined over 0.01–6 mM ATP-Mg^{2+}. Fig.1 shows that the Eadie-Hofstee plots representing v as a function of v/s were triphasic both with the isolated enzyme and with the membrane-bound enzyme. These results contrast with [1,2] in which biphasic patterns were obtained. This disagreement could be attributed to different experimental conditions. Here, ATPase activity was always determined holding the ATP/Mg^{2+} constant and equal to 1 in order to avoid the inhibitory effect of excess Mg^{2+} on the hydrolytic reaction [13,14].

The triphasic pattern of the Eadie–Hofstee plots suggests the existence of 3 catalytic sites in the ATPase with different affinity for the substrate. The existence of 3 different catalytic sites was also suggested after the observation that the hydrolytic activity of ATPase exhibited a maximum at 3 different pH-values depending on the substrate concentrations used [3]. Studies with analogs of ATP are also in agreement with the idea of a multiplicity of catalytic sites in mitochondrial ATPase [4,5].

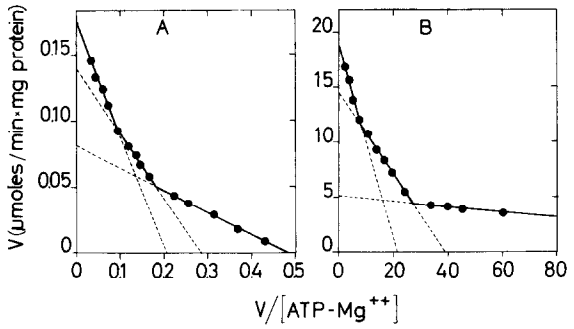

Fig.1. Eadie–Hofstee plots of the initial velocity of ATP-Mg^{2+} hydrolysis with isolated ATPase (B) and with the enzyme bound to membrane (A); 8 expt.

From the Eadie–Hofstee plots, K_m and V_{max} for each catalytic site have been calculated and the results are given in table 1. These values are of the same order as those found by others [2,15]. The K_m value of the high affinity site was higher in mitochondria than in the purified enzyme, whereas those of intermediate affinity were very similar in

Table 1

Kinetic parameters of mitochondrial ATPase, free and bound to membrane

	High affinity site		Intermediate affinity site		Low affinity site	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
F_1-ATPase	0.023	5.00	0.36	14.37	1.24	18.85
Membrane-bound ATPase	0.167	0.08	0.45	0.14	0.78	0.17
F_1-ATPase	K_i	K_a	K_i	K_a	K_i	K_a
KOCN	0.94		0.67		0.45	
KSCN	9.35		2.61		1.37	
DNP		0.95		0.33		0.18
CO_3H^-		35.95		6.75		3.20
Membrane-bound ATPase						
KOCN	0.86		0.55		0.20	
KSCN	5.77		1.17		0.68	
DNP		1.20		0.18		0.16
CO_3H^-		14.38		6.58		2.92

Velocity has been expressed as μmol ATP hydrolyzed . min^{-1} . mg $protein^{-1}$

K_m, K_i and K_a values are expressed as mM

both types of preparations. However, the K_m value of the low affinity site was higher in the free than in the membrane-bound enzyme. These differences might reflect a different conformation of the enzyme.

3.2. *Effect of anions on the hydrolytic activity of ATPase at different substrate concentrations*

The effect of inhibitors KOCN and KSCN, and of activators dinitrophenol and HCO_3^-, on the hydrolytic activity of ATPase has been studied over 0.06–4 mM substrate ATP-Mg^{2+}. Fig.2 shows the diagrams of $1/v$ as a function of inhibitor [KOCN] at fixed substrate concentrations both with the free enzyme and with mitochondria. It may be seen that KOCN behaved as a non-competitive inhibitor exhibiting 3 different K_i-values depending on the substrate concentrations used. The highest affinity for the inhibitor was obtained with the higher substrate concentrations used, whereas the lowest affinity was exhibited with the lower substrate concentrations. Similar studies were carried out with the inhibitors KSCN and with the activators DNP and bicarbonate. Values of K_i and K_a for the different inhibitors and activators are given in table 1. Both for activators and inhibitors, the highest affinities were exhibited at the highest substrate concentrations, and the lowest affinities at the lowest substrate concentrations. These results might indicate that the interaction of these different activators and inhibitors with a regulatory site or sites, affected in a different manner each one of the 3 catalytic sites of the enzyme. In [16], both activators and inhibitors were shown to compete for the same regulatory site or sites.

The slight differences in K_i and K_a values observed when inhibitors and activators were tested with the free and the bound enzyme could be tentatively explained as a consequence of different conformations possibly affecting the regulatory site or sites.

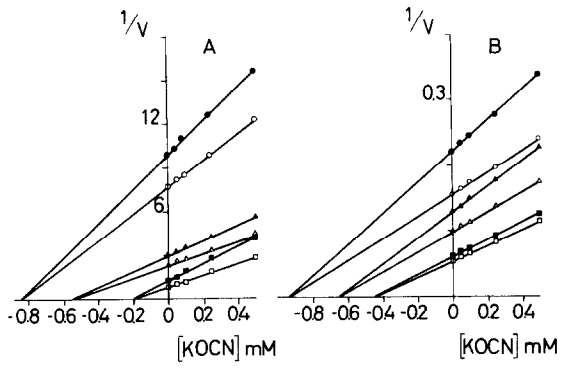

Fig.2. Effect of KOCN on ATPase activity at different fixed substrate concentrations: (A) isolated ATPase; (B) mitochondria. Substrate (mM): (●) 0.06; (○) 0.01; (▲) 0.3; (△) 0.6; (■) 2.5; (□) 4; 8 expt.

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