ABOLITION OF ANION-ACTIVATION OF MITOCHONDRIAL F_1-ATPase BY THE PARTIAL ADP-INDUCED HYSTERETIC INHIBITION

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1. Introduction

Several laboratories have observed the activation by anions of F_1-ATPase from mitochondria [1–7] or chloroplasts [8]. The mechanism of this activation is still a matter of discussion. On the basis of kinetic studies with mitochondrial ATPase of yeast, a strong influence of anions affecting the regulatory site for MgATP was reported and anions defined as allosteric ligands controlling the affinity of ATP to its hydrolyzing site [6]. In [7] the kinetics of the mitochondrial F_1-ATPase activity were studied at various temperatures with different ATP analogues. Anions were suggested not to affect the binding of nucleotides at regulatory sites but directly interfere with catalytic site(s) by promoting the release of the reaction products [7]. The latter explanation was invoked in [5].

In [9], after preincubation of the mitochondrial F_1-ATPase with ADP, ADP remained bound at regulatory site(s) and induced a 'hysteretic' inhibition which progressively developed in the presence of MgATP. This process brought the enzyme into a stable conformation of low specific activity.

This paper directly proves that anions do not modify the binding of ADP at regulatory site(s). Moreover, it will be shown that, when the enzyme has reached its stable conformation of low specific activity, the anions can no longer induce their characteristic activation. This suggests that, in this conformation, the anion binding site(s) is(are) no longer accessible to the external medium or that the activating effect of anions can no longer occur when the regulatory site(s) is(are) occupied by ADP.

2. Materials and methods

Nucleotides, phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim. Sodium bicarbonate, potassium chromate and 2,4-dinitrophenol were procured from Prolabo. Maleic acid and malonic acid were obtained from Sigma. [^{3}H]ADP (22 Ci/mmol) was purchased from Amersham.

Nucleotide-depleted F_1 was prepared from pig heart mitochondria as in [10]. It was stored frozen at −80°C in 100 mM Tris–H_2SO_4–5 mM EDTA–50% glycerol (pH 8.0) at 5 mg protein/ml. The ADP-induced hysteretic inhibition was measured as in [9,11] by preincubating the enzyme (1 mg protein/ml) in 50 mM Tris–H_2SO_4–10% glycerol–1.5 mM $MgSO_4$ (pH 8.0) containing 200 μM ADP unless otherwise indicated. Magnesium at 1.5 mM was sufficient to entrap the ADP in its regulatory site(s) [9]. After 20 min at 30°C a 0.5–1 μl aliquot was removed and diluted in 0.62 ml of the ATPase assay mixture. The latter was made of 50 mM Tris–H_2SO_4 buffer (pH 8.0) the activating anion as indicated below, 4 mM phosphoenolpyruvate, 0.3 mM NADH and 3.3 mM ATP plus 3.3 mM $MgSO_4$; this Mg^{2+} level being necessary to form the ATP–Mg complex, the enzyme substrate, at saturating concentration. The pH was adjusted again to 8.0 with KOH and 50 μg pyruvate kinase and 10 μg lactate dehydrogenase were added. The disappearance of NADH was recorded for several minutes at 340 nm except when chromate (315 nm) or 2,4-dinitrophenolate (300 nm) was present. The 'stable-inhibited rate' will be referred to as the rate measured 1 min after the addition to the ATPase

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assay medium of the assay preincubated with ADP (section 3).

The binding of [^{3}H]ADP was measured as in [9,11] after elimination of the free ADP by the filtration–centrifugation method in [12]. Protein concentration was estimated as in [13].

3. Results

Preincubation of F_1-ATPase with ADP followed by dilution in the ATPase assay mixture, containing an ATP-regenerating system, induced an inhibition which progressively developed with time. A stable inhibited rate was reached after ~1 min (fig.1A, upper curve). If ADP was omitted from the preincubation medium this effect was not observed (fig.1A, lower curve). As shown in [9], ADP is entrapped in regulatory site(s) different from the catalytic site(s) and not directly accessible to the external medium. The further addition of MgATP in the ATPase assay induces a conformational change that finally leads to a stable inhibition.

The presence of bicarbonate in the ATPase assay (fig.1B) increased 2.3-fold the rate of ATP hydrolysis of the control assay preincubated without ADP (fig.1B, lower curve). When the activity of the assay preincubated with ADP was measured in the presence of bicarbonate (fig.1B, upper curve), the initial rate was also enhanced as compared with that measured in the absence of bicarbonate. This activation slowed down during the first 1 min of the reaction as the hysteretic inhibition appeared. The bicarbonate-activation was completely abolished when the enzyme reached its stable inhibited conformation of low ATPase activity. It has been noticed that the lower curve (fig.1A) is never completely linear during the first seconds: the rate of the control measured in the absence of bicarbonate slightly increased during the first seconds and reached a stable value. In [14] preincubation of the enzyme with magnesium, in the absence of ADP, induced an inhibition that can be reversed. Thus the initial increase in the reaction rate observed in the absence of ADP (fig.1A) was due to the reversal of inhibition after dilution of the aliquot in the assay medium. The final stable value was the same as that of the control preincubated without magnesium (not shown). The inhibition by magnesium was no longer observed in the control measured in the presence of bicarbonate (fig.1B, lower curve). This was probably due to reversion of the magnesium-induced inhibition by the activating anion as observed in [15].

The absence of activation by bicarbonate of the stable-inhibited rate of ATP hydrolysis, induced by preincubation with ADP, was independent of the bicarbonate concentration (fig.2). In the same conditions, the control preincubated without ADP was activated by bicarbonate. A half-maximal effect was obtained with 2.4 mM bicarbonate.

As reported in [1–7], various anions are able to

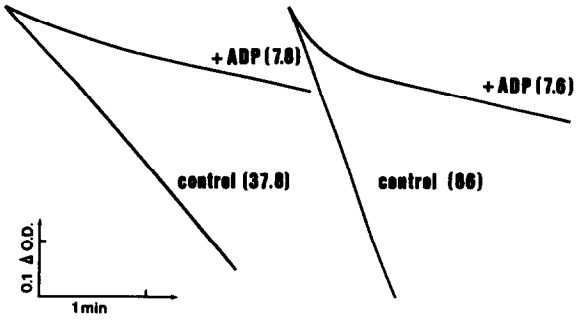

Fig.1. Loss of the bicarbonate-induced activation by preincubation with ADP. Nucleotide-depleted F_1 was preincubated with ADP as in section 2. The ATPase activity was measured on aliquots either in the absence (A) or in the presence (B) of 10 mM $NaHCO_3$; the values obtained from the slope of the linear portion of the recordings are indicated in parentheses. Results are expressed in units/mg protein (1 unit = 1 μmol ATP hydrolyzed/min). The controls were conducted under the same conditions except that ADP was omitted during the preincubation.

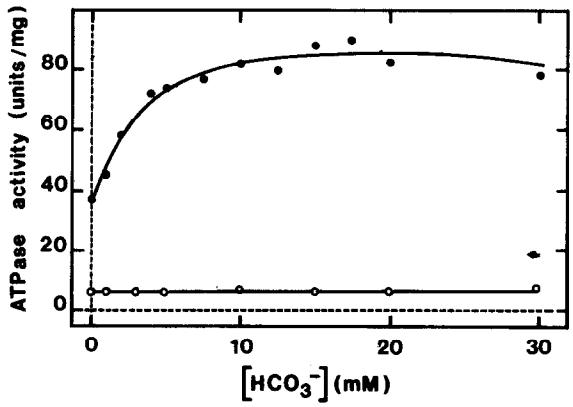

Fig.2. ATPase activity as a function of bicarbonate concentration after preincubation of the enzyme with or without ADP. The conditions were the same as in fig.1 for the control (•) or for the sample in the presence of ADP (○) except that bicarbonate in the ATPase assay varied as indicated.

Table 1
Effects of preincubation with ADP on the activation of ATPase activity by various anions[a]

Activating anion[b]	ATPase activity (units/mg)	
	Control	+ ADP
None	38 ± 5 (18)	7.8 ± 1.25 (6)
Bicarbonate		
2.4 mM	61	7.0
10 mM	86 ± 8 (11)	7.6 ± 1.2 (3)
Chromate		
0.6 mM	62	8.7
4 mM	88	8.5
Maleate		
13 mM	60	6.0
50 mM	88	6.0
Malonate		
7 mM	49	6.0
25 mM	62	6.6
2,4-dinitrophenolate		
0.125 mM	51	6.4
0.4 mM	61	7.2

[a] Preincubation with or without ADP was as in fig.1. Samples were removed to measure ATPase activity in the presence of the indicated anion

[b] The lower anion concentration used produced the half-maximal activation whereas the higher concentration induced the maximal activation

increase the ATPase activity of F_1 with different efficiencies. In the conditions used here (table 1), bicarbonate, chromate and maleate produced a maximal activation higher than malonate and 2,4-dinitrophenolate. The half-maximal activation was induced by a concentration of 2,4-dinitrophenolate much lower (0.125 mM) than that of maleate (13 mM). However, whatever the nature or the concentration of the anion was, the same stable-inhibited rate was observed after preincubation of the enzyme with ADP. Indeed, the values obtained in the presence of the different anions were not significantly different from that measured in the absence of activating anion.

When bicarbonate was present in the preincubation medium but omitted in the assay medium, its concentration in the assay was too low to induce any activation. Fig.3 shows that the presence of bicarbonate in the preincubation medium modified neither the binding of [^{3}H]ADP to the enzyme nor the concomitant inhibition: there was no significant difference between the assays preincubated with bicarbonate (open symbols) or without bicarbonate (closed symbols). The bicarbonate-induced activation of the ATPase activity decreased when the ADP concentration increased in the preincubation medium (circles). This activation completely disappeared at 50 μM ADP which corresponds approximately to the maximal ADP binding ([9], squares).

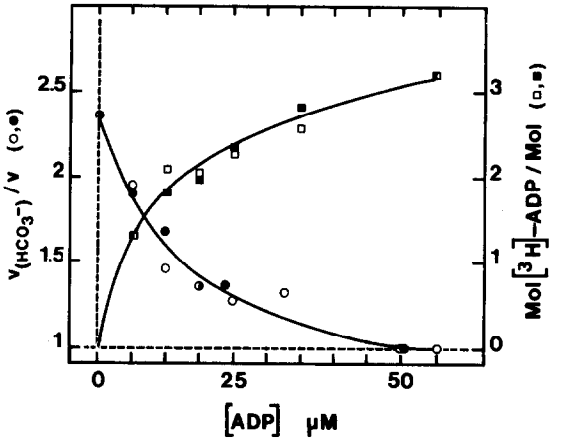

Fig.3. Inefficiency of bicarbonate pretreatment of the enzyme in modifying the ADP-induced hysteretic inhibition and concomitant [^{3}H]ADP binding. The enzyme was pretreated (○,□) or not (●,■) for 15 min with 15 mM $NaHCO_3$ before the addition of the indicated concentrations of [^{3}H]ADP (100 dpm/pmol). The stable-inhibited rate of ATP hydrolysis was measured in the presence (v_{HCO_3}-) or the absence (v) of 10 mM $NaHCO_3$ in the ATPase assay mixture (○,●). The binding of [^{3}H]ADP (□,■) was estimated after the elimination of free ADP by filtration–centrifugation [9,11]. The M_r-value of F_1-ATPase was taken as 380 000 [16].

4. Discussion

These results clearly demonstrate that the presence of anions modify neither the binding of ADP at regulatory site(s) nor the ADP-induced hysteretic inhibition, which excludes a competition between anions and ADP binding at regulatory site(s). The regulatory site(s) can be occupied either by ADP or ATP [9]. On the basis only of studies of the rate of ATP hydrolysis, the effects of anions on nucleotide binding and on the ATPase activity are difficult to distinguish. This is perhaps why a competition between anion and nucle-

otides at regulatory sites was proposed to explain the results in [6].

Binding of ADP at regulatory site(s) and further MgATP hydrolysis induce a new conformation of the enzyme, insensitive to anion activation. Since the abolition of anion activation is not immediate but develops as the conformational change is established, the curvature of ATP hydrolysis kinetics induced by the assay preincubated with ADP is more apparent in the presence of activating anions in the ATPase assay. Therefore activating anions are good probes to monitor the conformational change related to the hysteretic behavior of the enzyme. Other experiments will be necessary to prove whether or not anions can bind when the enzyme has reached the ADP-dependent stable conformation of low specific activity. In any case the anions are no more able to induce their characteristic activation whether they bind or not to the ADP-inhibited conformation. It has been shown that the stimulation of MgATP hydrolysis by anions was limited with submitochondrial particles [4,5]. This might suggest that the conformation of the enzyme exhibiting a low specific ATPase activity after induction of hysteretic inhibition is closer to that existing in situ in the membrane than the conformation of high specific activity obtained after purification of F_1-ATPase.

Acknowledgements

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