MASKING OF CO-OPERATIVITY OF NUCLEOTIDE SITES IN PIG HEART MITOCHONDRIAL ATPase (F_1) BY HEATING*

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1. Introduction

Although the soluble mitochondrial ATPase (F_1) [1] is an enzyme complex constituted of 5 polypeptides with different stoichiometry, most studies report linear Michaelis and Menten kinetics [2–4] for ATP hydrolysis and its inhibition by ADP. Kinetic studies did not reveal several nucleotide sites in purified F_1-ATPases** except in the case of rat liver ATPase by Ebel and Lardy [5] who observed a concave curvature of reciprocal plots of initial velocity with MgATP as the variable substrate, in the absence of other anions.

In a previous paper [6], we applied the Senior and Brooks procedure [7] to purify pig heart mitochondrial ATPase (F_1). The resulting preparation behaved mainly as that of beef heart in other laboratories (cf reviews from Senior [8], Pedersen [9] and Penefsky [10]).

The present work shows that with soluble pig heart mitochondrial ATPase (F_1), prepared without the heating step, interaction between ATP and ADP sites can be demonstrated by kinetic studies: reciprocal plots of initial velocity with ATP as the variable substrate, become curved with increasing ADP concentrations (Hill coefficient from 1 to 2.1). This effect is not observed if the F_1 has been heat treated. Besides, this heating step, that does not eliminate any contaminating protein, induces a conformational change that increases the ATPase specific activity and diminishes the number of titrable thiol groups.

2. Material and methods

Pig heart mitochondria were obtained at 0–4°C as previously described [11]. The Senior and Brooks procedure [7] was followed to purify F_1 with minor modifications, as reported earlier [6]. The last step that consists of heating F_1 (10 mg protein per ml of buffer containing 40 mM Tris–SO_4, 1 mM EDTA, 4 mM ATP, pH 7.5) for 2 min at 65°C, then cooling it quickly at 25°C in a water bath and spinning it down in an Eppendorf centrifuge for 4 min to remove the precipitate, was performed or omitted. Protein contents were estimated using the Lowry procedure [12] and gel electrophoresis in sodium dodecyl sulphate was made using 10% polyacrylamide gels [13]. ATPase activity was determined colorimetrically: the assay mixture routinely contained 42 mM Tris(maleate), 3.3 mM ATP, 2.5 mM $MgSO_4$, pH 8.0; after 3 min incubation at 30°C, 1.3 μg enzyme solution was added. Final vol was 0.6 ml. The reaction proceeded for 1 min and was stopped by adding 0.5 ml ice-cold 10% trichloracetic acid. Inorganic phosphate released was estimated by the Sumner method [14]. Results are expressed as μmol P_i released per min per mg protein.

Thiol groups were estimated spectrophotometrically by the Ellman procedure [15] using DTNB or CPDS. The latter can react with thiols in two different ways

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** *Abbreviations:* DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); CPDS, carboxy pyridine disulfide or 6,6′-dithiodinicotinic acid; F_1, soluble mitochondrial ATPase.

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[16], both leading to the formation of disulphide bonds with a concomitant release of thione or 6-mercaptonicotinic acid that shows a maximum absorption at 344 nm with a molar extinction coefficient of 10^4. F_1 prepared without the heating step was kept at 0–4°C in 40 mM Tris–sulphate, 1 mM EDTA, 2 mM ATP, 55% saturated $(NH_4)_2SO_4$, pH 7.5. F_1 stored after the heating step was kept in the same medium. Before estimating the accessible thiol groups, F_1 was submitted or not to the heating step; it was always charcoal treated to remove any free nucleotide [17]. After centrifugation of the F_1 ammonium sulphate suspension, the protein precipitate was dissolved in 0.4 ml 0.2 M phosphate (K) buffer, 1 mM EDTA, pH 7.5 and precipitated again by adding 0.9 ml of saturated ammonium sulphate solution containing 1 mM EDTA, pH 7.5. After centrifugation, the precipitate was dissolved again in phosphate buffer; 2 mg of activated charcoal were added. After mixing, charcoal was removed by centrifugation. F_1 was precipitated again by ammonium sulphate from the supernatant fluid. After centrifugation, the pellet was finally dissolved in 0.55 ml 10 mM Tris–Cl, 1 mM EDTA, pH 7.5, ± 2 mM ATP. Protein determination was conducted on 50 µl aliquots. DTNB or CPDS were added directly in the assay cuvette and changes in optical density were followed as a function of time at 412 nm (DTNB) or 344 nm (CPDS) against control cuvettes, identical except that they were free from F_1.

CPDS was obtained from Newcell Biochemicals, nucleotides from Boehringer; all other reagents were of the purest available grade from Sigma Chemical Co., Prolabo or Merck.

3. Results and discussion

3.1. *Influence of heating on ATPase activity and subunit composition*

The last purification step of F_1 used by Senior and Brooks [7] and other laboratories [1,17] consists of heating the enzyme in the presence of ATP. When this treatment was applied to pig heart mitochondrial F_1, the total ATPase activity was increased by 5% in each experiment and the specific activity raised from 92 units/mg protein to 125 units/mg protein (average of five different preparations). One could expect that this step would eliminate any remaining F_1 protein inhibitor. However, in this preparation, as it was observed for the Brooks and Senior preparation [18], the unheated enzyme did not inhibit the heated one; on the other hand, this protein inhibitor could not be detected in unheated F_1 when assayed according to Warshaw et al. [19]; moreover, in sodium dodecyl sulphate gel electrophoresis (fig.1), only five different components could be detected respectively with apparent mol. wts of 58 700 ± 900, 54 700 ± 700, 28 600 ± 1400, 12 700 ± 300, 8700 ± 600 (average of five different preparations ± standard error of the mean). These subunit mol. wts. have been measured according to Weber and Osborn [20]. No band of mol. wt 10 500 corresponding to the beef heart F_1 protein inhibitor could be detected. Gel electrophoresis profiles were not modified by putting on the gels either the active enzyme before or after heating or the inactive residue obtained during the heating step. Therefore, this step did not eliminate any protein contaminant but increased the catalytic activity of the enzyme. This may have come from a conformational change of F_1.

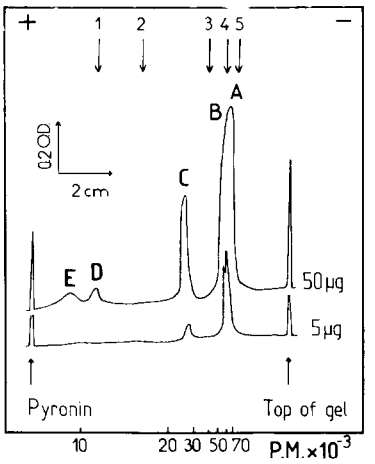


Fig.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of pig heart F_1 (heated or unheated). Conditions as described by Catterall et al. [13]; the subunits mol. wts. were determined as shown by Weber and Osborn [20] using cytochrome *c* (1), myoglobin (2), egg albumin (3), beef liver glutamate dehydrogenase (4) and bovine serum albumin (5) as marker proteins.

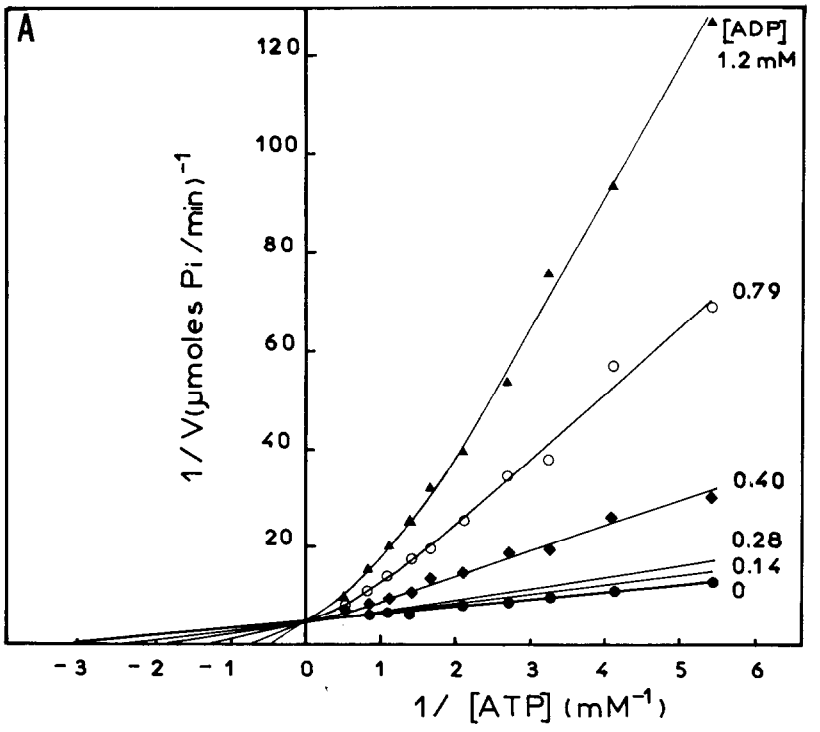
3.2. *ADP inhibition kinetics of heated and unheated F_1*

Further evidence for this conformational change of F_1 was obtained by studying inhibition kinetics of F_1 by ADP. In all experiments, the ratio nucleotides (ATP + ADP)/Mg^{2+} was maintained at 1.33 [6]; in the absence of Mg^{2+}, no ATPase activity could be detected. Fig.2 compares initial rates of ATP hydrolysis in the presence of increasing ATP and ADP concentrations for unheated and heated F_1. In both cases, ADP behaved as a competitive inhibitor. With heated F_1, reciprocal plots of initial velocities with varying ATP concentrations were linear whatever the ADP concentration was; with unheated F_1, these plots became more and more curved when the fixed ADP concentration increased. The Hill coefficients for ATP (table 1) remained near 1 with heated F_1 whatever the ADP concentration was; with unheated F_1 it increased from 1 to 1.34 when ADP concentration increased from 0 to 1.2 mM. With heated F_1 the Hill coefficient for ADP (table 2) did not change significantly from 1–1.1 by decreasing ATP concentration. On the contrary, with the unheated enzyme, the Hill coefficient for ADP increased from 1.3 to 2.1 by decreasing ATP concentration from 1.84 mM to 0.18 mM; reciprocal plots of initial rate of ATP hydrolysis as a function of ATP concentration are linear in the absence of added ADP and become non linear with increasing ADP concentrations. Therefore, the presence of ADP made apparent a positive cooperativity between nucleotide sites. If the ratio nucleotide

Table 1
Hill coefficients of heated and unheated F_1 for ATP at fixed ADP concentrations

ADP (mM)	Hill coefficient	
	heated F_1	unheated F_1
0	1.0	1.0
0.14	0.98	0.94
0.28	0.94	0.94
0.40	0.94	1.06
0.55	0.96	
0.79	0.95	1.26
1.20	1.0	1.34

Experimental conditions as described in fig.2.



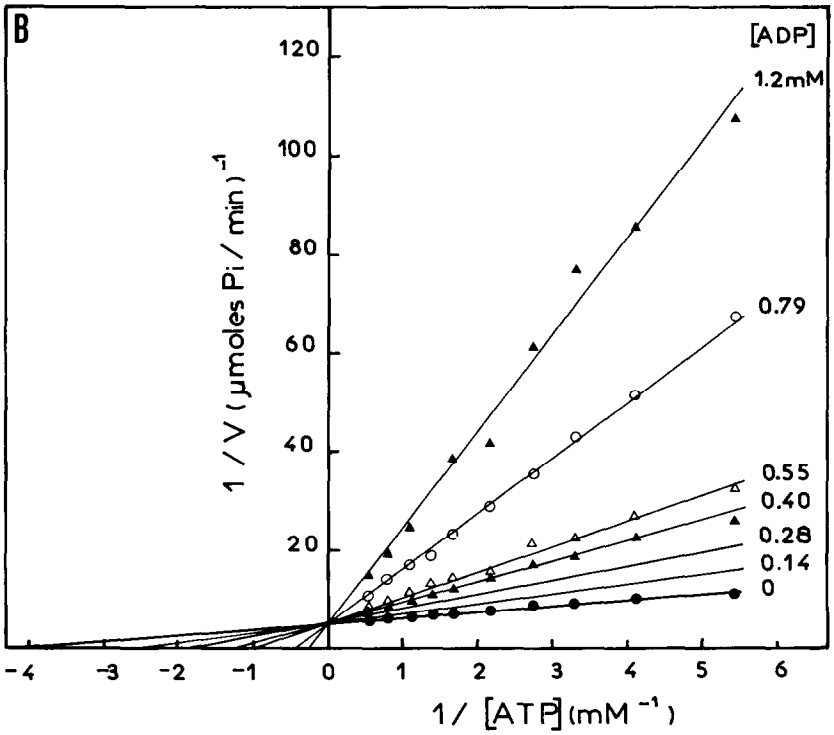


Fig.2. ADP inhibition of unheated pig heart F_1 (fig.2A) and heated pig heart F_1 (fig.2B). To 0.5 ml 50 mM Tris (maleate) pH 8.0 were added aliquots of 10 mM ATP–7.5 mM $MgSO_4$, pH 8.0 and aliquots of 30 mM ADP–22.5 mM $MgSO_4$, pH 8.0 (therefore the ratio nucleotide/Mg^{2+} was always 1.33); reaction was initiated by 2 µg unheated F_1 or by 1.4 µg heated F_1 (0.8 ml final vol) and stopped after 1 min by adding 0.5 ml ice-cold 10% trichloracetic acid. Released inorganic phosphate was estimated by the Summer method.

(ATP)/Mg^{2+} was lowered by adding $MgSO_4$ instead of Mg-ADP, no change in the rate of ATP hydrolysis was observed; therefore, the cooperative effects did not appear related to Mg^{2+} interactions in the studied Mg^{2+} concentration range.

Table 2
Hill coefficients of heated and unheated F_1 for ADP at fixed ATP concentrations

ATP (mM)	Hill coefficient heated F_1	unheated F_1
0.18	1.13	2.1
0.31	1.11	2.0
0.92	1.06	1.6
1.22	1.08	1.6
1.84	1.04	1.3

Experimental conditions as described in fig.2.

These co-operative effects were not detected in studies with beef heart F_1 [2,3], probably because the authors used a heating step in their preparation. The Ebel and Lardy [5] observations on rat liver F_1 are of a different nature since the Hill coefficients they obtained were lower than one, in the absence of anion and equal to one, in the presence of an added anion. The experiments were performed here in the presence of maleate which allowed a high enzyme activity. Although the exact mechanism of interaction of nucleotides remains to be understood, Hill coefficients higher than two suggest that there are more than two nucleotide binding sites; the observed competitions would imply that ATP and ADP could compete to occupy them or they could reflect interactions between different types of sites. These observaitons may be related or not to the findings of Harris et al. [17] who reported five tight binding

sites for ATP and ADP and to the findings of Garret and Penefsky [21] and Penefsky [22] who found two binding sites for an analog of ATP and probably one additional binding site for ADP, using beef heart mitochondrial F_1.

3.3. *Thiol reactivity of unheated and heated enzyme in the presence or the absence of ATP*

The conformational change induced by heating also modified the thiol groups reactivity of F_1. Reaction of F_1 with thiol reagents: DTNB or CPDS, was followed spectrophotometrically for about 15 min, respectively at 412 nm or 344 nm. In the presence of ATP, the unheated enzyme could react quickly with both reagents. A plateau was reached within one minute with DTNB and slightly more than one thiol group reacted per mole of enzyme (assumed mol. wt.: 385 000); with CPDS, the plateau was reached after four min. when more than two thiol groups had reacted. This reaction of F_1 with thiol reagents did not modify ATPase activity.

Table 3 compares these results with those obtained with heated F_1 in the presence or the absence of ATP; F_1 was previously treated with charcoal, to eliminate any free nucleotide. In the presence of 2 mM ATP, the number of titrable thiol groups was always higher. If F_1 had been heated just before use, (highest specific activity) DTNB could titrate only about 0.5 –SH groups instead of 1.4 for the unheated enzyme in the presence of ATP. Almost no –SH groups were detected in the absence of ATP, while 1.04 were estimated in the unheated enzyme. CPDS could react with one thiol group of heated F_1 instead of 2.28 of the unheated enzyme in the presence of ATP and with 0.22 thiol group instead of 1.05 of the unheated enzyme in the absence of ATP. If F_1 had been heated and kept as a suspension in ammonium sulphate at 0–4°C for several months, it behaved about the same way as the enzyme before heating and revealed a number of titrable –SH similar to those of the unheated F_1. During this storage period, the specific ATPase activity had decreased, becoming close to the value of the enzyme before heating. Heating again this preparation diminished the number of titrable thiol groups and increased the specific activity up to the value obtained for the heated enzyme Therefore, if the thiol groups accessibility of DTNB and CPDS gives a reliable picture of the structure of F_1, the conformational change induced by heating would be slowly reversible at 0–4°C when F_1 is kept as an ammonium sulphate suspension.

The increase in titrable F_1 SH groups observed in the presence of ATP, is seen with both the heated or the unheated enzyme. Senior [23] reported the diminution of titrable –SH groups of beef heart F_1 after heating but did not detect a difference in the presence of ATP; however his enzyme was not char-

Table 3
Thiol groups of heated and unheated pig heart F_1 in presence or absence of ATP

Enzyme heating	Thiol reagent	nmoles titrable –SH/nmole F_1			
		+ 2 mM ATP	n[a]	No ATP	n[a]
No	DTNB	1.38 ± 0.22	4	1.04 ± 0.14	4
	CPDS	2.28 ± 0.23	4	1.05 ± 0.07	5
Just before titration	DTNB	0.48 ± 0.05	4	0.09	2
	CPDS	0.99 ± 0.05	3	0.22	2
5 months before titration	DTNB	1.07 ± 0.19	4	0.88	1
	CPDS	1.87 ± 0.23	4	1.14	1

Results are expressed as the mean of n experiments followed by the standard error of the mean. The molecular weight of pig heart F_1 is assumed to be 385 000 [6]. Experimental conditions of titration have been described in material and methods.

[a] n = number of different preparations

coal treated and he used DTNB that did not give a difference as large as CPDS in our experiments. The weaker reactivity of DTNB as compared to CPDS could come from the NO_2 group of DTNB that modifies charge and steric hindrance. Further studies are in progress to elucidate the rôle of –SH groups in ATPase mechanism.

It is possible that heating provokes a transition of part of F_1 from an inactive form into an active form. Moyle and Mitchell [24] suggested for F_1 from beef heart or rat liver, the existence of both an inactive and active form. The number of accessible thiol groups of the active form would be lower than those of the active form; the slow recovery of the heated enzyme (Table 3) would be in agreement with Moyle and Mitchell hypothesis i.e. 'the active/inactive state transition involves some slow change of conformation of the individual subunits of F_1 or some change of packing of the subunits or both'. However, the cooperative effects of nucleotide sites require more than a slow transition between two structures which would not explain the effects observed in kinetic experiments measuring initial rates of ATP hydrolysis without preincubation of the nucleotides with F_1.

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