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Pre-steady-state properties of bovine heart mitochondrial ATPase: a nucleotide-dependent H^+ burst

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The transient kinetics of bovine heart mitochondrial ATPase (F_1) depleted of loosely bound nucleotides were observed. The activation process which was shown as a lag time before steady-state hydrolysis observed previously (Clark et al. (1984) Arch. Biochem. Biophys. 233, 378–392) was preceded by a proton burst when F_1 was stripped of its loose nucleotides. 5′-Adenylylimidodiphosphate (Ado *PP*[NH]*P*) or MgATP binding is shown to cause proton release. Maximum proton release per F_1 free of loosely bound nucleotides is observed with MgATP. Modification with NBD-CL of F_1 that was nucleotide-depleted eliminated the proton burst, which suggests that the modified tyrosine (i.e., in the catalytic subunit) is directly involved in the release of protons.

Introduction

The transient kinetic properties of the hydrolysis reaction by soluble bovine heart mitochondrial ATPase, F_1, have been described extensively [1–5]. These studies are consistent in that they all conclude the enzyme undergoes an activation process before steady-state hydrolysis occurs. The activation process is manifested in a lag time which lasts for many catalytic turnoverers.

This paper reports a new observation of the pre-steady-state reaction of F_1. The lag in hydrolysis of MgATP by F_1 is preceded by a proton burst when the enzyme is treated to remove the loosely bound nucleotides. This transient release of protons was investigated using phenol red as indicator, in the hopes of obtaining further insight into the detailed sequence of events in the mechanism of regulation and catalysis of ATP synthesis and hydrolysis.

Abbreviations: Ado*PP*[NH]*P*, 5′-adenylylimidodiphosphate; NBD-CL, 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid.

Experimental procedures

Materials and Methods

Bovine heart mitochondrial ATPase (F_1) was prepared by the method of Spitsberg and Blair [6]. Phenol red, phosphoenolpyruvate, NADH, ATP, Ado*PP*[NH]*P*, ADP and NBD-CL were obtained from Sigma (St. Louis, MO). LDH (bovine heart) and pyruvate kinase (rabbit muscle) were purchased from Sigma as crystalline suspensions in $(NH_4)_2SO_4$ solution. All other reagents were analytical grade. Glass distilled, deionized water was used throughout.

Steady-state enzyme assays were conducted on a GCA/McPherson EU-700 series double beam spectrophotometer. Stopped-flow experiments were run using an Aminco DW-2A dual-wavelength spectrophotometer equipped with a temperature-controlled stopped-flow mechanism with an opti-

cal pathlength of 1.0 cm (American Inst. Co.) [5]. Dead time of the instrument was determined to be 4.5 ms in experiments monitoring the reduction of $K_3Fe(CN)_6$ by ascorbate [7]. All data acquisition and calculations were carried out on a Horizon II microcomputer (North Star Computers, Inc.) [8].

A coupled enzyme assay was used to determine the specific activity of F_1. The disappearance of NADH was observed by monitoring the change in absorbance at 339.0 nm (pH 7.7). Final concentrations of the various constituents of the assay were 20.0 mM Tricine, 5.0 mM ATP, 10.0 mM Mg^{2+}, 1.47 mM phosphoenolpyruvate, 0.42 mM NADH, 2.24 units/ml lactate dehydrogenase activity and 1.92 units/ml pyruvate kinase activity. Varying amounts of F_1 (10^{-7}–10^{-9} M) were used.

Preparation of enzyme

Native enzyme was obtained by the method described [6] and resuspended in a 4.0 mM ATP and 1.0 mM EDTA solution. This solution was saturated with ammonium sulfate to 60% and stored at 4°C. Just before use the F_1 was centrifuged for 10 min at 10000 rpm in a Sorvall RC-5 Superspeed Refrigerated centrifuge; the pellet was dissolved in 40 mM Tris-SO_4/1 mM EDTA buffer at pH 7.77, and then mixed. The enzyme was precipitated 4 times with 65% $(NH_4)_2SO_4$ to remove loosely bound nucleotides as described [9]. Enzyme concentration was determined by the Bradford method [10] using Bio-Rad protein assay dye reagent concentrate and bovine serum albumin as standards. This precipitated modified F_1 will be referred to as 'free F_1'. The free F_1 had an A_{280}/A_{260} ratio of 1.79–1.83, whereas native F_1 had an A_{280}/A_{260} ratio of 1.52. According to Tamura and Wang [11] nucleotide content of free F_1 was approx. 1.0 (ADP + ATP)/F_1 with this absorbance ratio.

Chemical modification of enzyme

Free F_1 was dissolved in a buffer consisting of 200 mM sucrose/4 mM EDTA/50 mM Bes (pH 7.77). The reaction with NBD-Cl was initiated by addition of 50 μM NBD-Cl (dissolved in ethanol) to 2 ml of solution (3–4 mg/ml) as described [12]. Light was avoided as to minimize decomposition of NBD-Cl. The inactivation of free F_1 was followed by the steady-state coupling activity assay. When the inactivation reached the level desired, the NBD-Cl modification of free F_1 was terminated by addition of 2 vols. of cold saturated ammonium sulfate and then centrifuged. A standard of F_1 treated with ethanol was used as the control.

Stopped-flow assay and data transformation

Proton release is observed during the hydrolysis of ATP to ADP and inorganic phosphate. The hydrogen ions released into the medium at the pH and p*Mg*, at which these investigations took place was monitored by observing the change in absorbance of phenol red (a pH-sensitive dye). Properties of phenol red, reactions conditions, proton stoichiometry and formulations of reaction buffers have been described previously [5].

After an experiment had begun, its progress was followed by observing the decrease in absorbance of 559.0 nm relative to 650.0 nm. This decrease in absorbance was caused by a release of protons causing a change in the ionic form of phenol red. The time vs. absorbance data was then stored on magnetic diskettes for future reference. Each experiment was repeated 5 times and averaged.

Fig. 1 shows a typical experimental record with free F_1. The initial pH was calculated from the absorbance reading at the start of the reaction. The dashed lines in Fig. 1 demonstrates the method

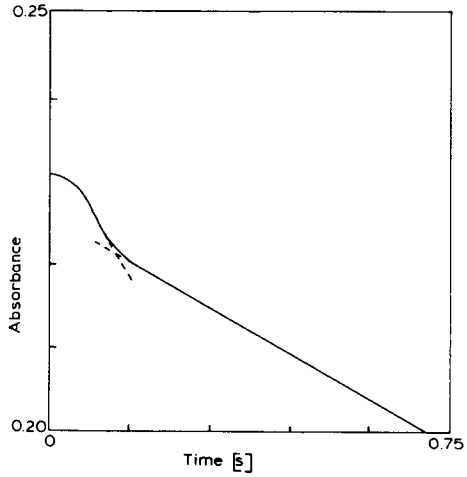

Fig. 1. A typical plot of absorbance vs. time for a reaction in which nucleotide-depleted F_1 was mixed with MgATP. The concentration of free F_1 in this experimental run was 47.8 nM. Concentrations of ATP and Mg^{2+} were 1.0 and 5.0 mM, respectively. Final concentration of phenol red is 10.0 μM.

used to calculate the final absorbance for the proton burst. This consists of extrapolating the steady-state rate and burst rate. The intersection of the two extrapolated lines is the final absorbance used to calculate the protons released in the burst. The final absorbance was corrected for by subtacting the absorbance change from hydrolysis in native F_1 under the same experimental conditions.

The change in absorbance is related to protons released by F_1 plus the buffer capacity of the system. The buffer capacity of the system was determined to be dependent on protons absorbed by the nucleotides and phenol red. Table I shows the initial and final concentrations of each species in the reaction. These concentrations were calculated by solving the nonlinear differential equations shown previously [5,13]. The experimental conditions in Table I were the same as in Fig. 1. The concentrations of protons absorbed by phenol red were also calculated using these absorbance readings and applying Beer's law. The absorptivity coefficient of phenol red used was $5.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 559.0 nm. After calculation of the concentrations of the two forms of phenol red, the pH of the reaction was calculated using the Henderson-Hasselback equation. Other than $HATP^{-3}$, $MgHATP^{-1}$, and phenol red, the buffer capacity of the system was insignificant at the pH where the reaction was carried out. From this, the change in absorbance was related to the moles of protons/mole of free F_1 released during each experiment. The concentration of enzyme used showed to be insignificant as a buffer in that the titration curve of Mg, ATP, and phenol red (under conditions identical to experimental conditions) were the same with and without 200 nM free F_1.

Results

The lag in MgATP hydrolysis by bovine heart mitochondrial ATPase (F_1) first observed by Reckenwald and Hess [2,3] was preceded by a proton burst when loose binding nucleotides were removed from F_1 (free F_1) (see Fig. 1). This proton burst is not observed with native F_1 as shown in Fig. 2. The early burst rate is apparent before the steady-state rate is reached. Experiments were performed to determine the cause of the proton burst.

Since the proton burst was not observed with native F_1 and only free F_1, the burst could reasonably be due to phenol red binding to enzyme depleted of loosely bound nucleotides. Two separate experimental controls show this not to be the case. First, the steady-state velocity of free F_1 was determined with and without phenol red. No change in ATPase activity was observed between F_1 with and without phenol red. Second, the absorption spectra of phenol red with and without F_1 were the same.

TABLE I

INITIAL AND FINAL CONCENTRATIONS OF SPECIES IN A STOPPED FLOW PROTON BURST EXPERIMENT

These concentrations (M) were determined as described [5,13]. Total concentrations of species were: phenol red (10 μM), magnesium (5.0 mM), ATP (1.0 mM). In this particular experiment the concentration of free F_1 was 47.8 nM. Therefore, 3.03 protons/free F_1 were released.

Species	Initial concentration (M)	Final concentration (M)	Change in concentration (M)
$[H^+]$	$2.55026 \cdot 10^{-8}$	$2.59206 \cdot 10^{-8}$	$4.18 \cdot 10^{-10}$
[Phenol Red]	$5.55338 \cdot 10^{-6}$	$5.59349 \cdot 10^{-6}$	–
$[Phenol\ Red^-]$	$4.44661 \cdot 10^{-6}$	$4.40651 \cdot 10^{-6}$	$4.01 \cdot 10^{-8}$
$[Mg^{2+}]$	$4.00786 \cdot 10^{-3}$	$4.00789 \cdot 10^{-3}$	–
$[MgATP^{-2}]$	$9.87555 \cdot 10^{-4}$	$9.87451 \cdot 10^{-4}$	–
$[MgHATP^{-1}]$	$4.58339 \cdot 10^{-6}$	$4.65802 \cdot 10^{-6}$	$7.463 \cdot 10^{-8}$
$[ATP^{-4}]$	$6.04923 \cdot 10^{-6}$	$6.04855 \cdot 10^{-6}$	–
$[HATP^{-3}]$	$1.81261 \cdot 10^{-6}$	$1.84211 \cdot 10^{-6}$	$2.95 \cdot 10^{-8}$
			Sum: $1.446 \cdot 10^{-7}$

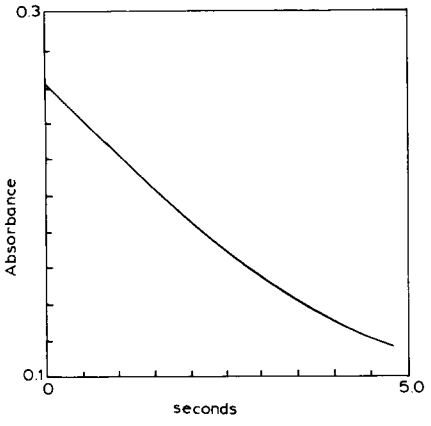

Fig. 2. A typical experimental record for a stopped flow ATPase experiment. This particular experiment used 0.5 mM ATP, 5.0 mM Mg and 27 nM F_1.

To determine if the proton burst was due to a rapid hydrolysis of substrate, Ado*PP*[NH]*P* (a nonhydrolyzable ATP analog) was used as a substrate substitute. When free F_1 was mixed with Ado*PP*[NH]*P*, initial proton release was observed. When free F_1 concentration was kept constant and Ado*PP*[NH]*P* concentration varied, the amount of proton release increased. Fig. 3 is a double reciprocal plot showing free F_1/H^+ released vs. 1/[Ado*PP*[NH]*P*]. Positive cooperativity associated with the appearance of the proton burst is observed. Extrapolation of the line to infinite Ado*PP*[NH]*P* concentration shows a maximum release of 1.0 mol protons per mole of free F_1. If it is assumed that Ado*PP*[NH]*P* is not hydrolyzed by F_1, these results indicate that the

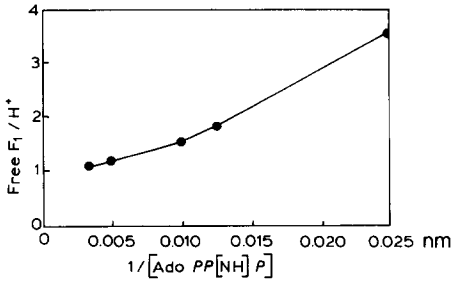

Fig. 3. A double reciprocal plot of proton released/free F_1 vs. Ado*PP*[NH]*P* concentration. Extrapolation of the data to infinite Ado*PP*[NH]*P* concentration indicates a maximum of one proton released per free F_1. In this experiment, free F_1 and magnesium concentrations were 88.6 nM and 5.0 mM, respectively.

proton burst is not turnover-dependent. This result shows that the proton burst is not caused by an initial burst in ATP hydrolysis activity, but is a result of binding. It must be noted that the burst, while probably not due to hydrolysis of Ado*PP*[NH]*P* in solution, could be due to the hydrolysis of tightly bound ATP not removed by the ammonium sulfate precipitation step.

Since ADP is a product of the ATPase reaction, it could alter the pre-steady-state proton burst. Therefore the effects of ADP on the proton burst were examined. Two experiments were performed. In the first experiment, ADP was mixed with the substrate MgATP before being introduced to free F_1. In the second experiment, MgADP was incubated with the free F_1 before being introduced to MgATP. Both experiments indicate that the proton burst decreases with increasing ADP concentrations. The size of the burst, even though decreased, was not eliminated. It was also observed that increasing concentrations of ADP mixed with enzyme decreased the steady-state hydrolysis rate.

The observations that the burst decrease with MgADP concentrations incubated with enzyme or substrate, indicate that metal-nucleotide complex is necessary, at least in part, for appearance of the proton burst. The fact that MgADP reduces the size of the proton burst may be due to one of several factors. Firstly, it could be that incubation with MgADP with free F_1 causes it no longer to be without loosely bound nucleotides. Secondly ADP could cause the burst to occur (which it does, data not shown), thereby reducing the burst upon addition of ATP. Thirdly, MgADP could be filling a specific site necessary for the MgATP-caused burst. At present any of these three possibilities is difficult to eliminate.

Free F_1 concentrations were varied to determine if any correlation with the proton burst existed. The steady-state hydrolysis rate increased linearly with increasing concentrations of free F_1. In all experiments, an initial delay of approx. 30 ms was observed followed by the burst of protons. The size of the proton burst (1.29 protons/free F_1) was independent of the concentration of free F_1. In these experiments the concentration of ATP and Mg^{2+} was 0.5 and 5.0 mM, respectively.

Since MgATP is the substrate of F_1-ATPase, it

was necessary to examine the burst of protons of free F_1 as the concentration of ATP is varied. ATP concentrations varying from 0.1 to 3.0 mM were used. Table II shows that as ATP concentrations increase, the proton released per free F_1 increase. Fig. 4 is a double reciprocal plot of free F_1/H^+ vs. 1/ATP. Fig. 4 shows negative cooperativity in the proton burst when ATP is used as substrate with free F_1. This is in contrast to that observed when protons were released by free F_1 upon the addition of Ado*PP*[NH]*P*. The maximum amount of H^+/mole of free F_1 released with MgATP is three compared to one with Ado*PP*[NH]*P* (Fig. 3). This shows that the hydrolyzable substrate induces a greater proton release than the non-hydrolyzable analog. This observation suggests that the protons released are, at least in part, associated with the catalytic subunit.

Several F_1 preparations have been shown to catalyze a very low rate of hydrolysis in the absence of any divalent cation [14,15], but it is generally believed that this enzyme requires a divalent cation for rapid catalysis. Therefore, the effect of varying magnesium concentration on the proton burst was investigated. Two experiments were conducted. In the first experiment magnesium was introduced to the free F_1 at the same time as ATP (i.e., during the mixing process). In the second experiment magnesium was incubated with the enzyme before being mixed with MgATP.

The experiment in which Mg^{2+} (1–5 mM) was mixed with ATP before being introduced to free F_1

TABLE II

AMOUNT OF $[H^+]$/[FREE F_1] VS. ATP CONCENTRATION

These data were calculated from an experimental series in which free F_1 concentration was 47.8 nM and Mg^{2+} was 5.0 mM.

[ATP] (mM)	$[H^+]$/[Free F_1]
3.0	2.97
2.0	2.70
1.0	2.60
0.8	1.81
0.5	1.25
0.2	0.67
0.1	0.5

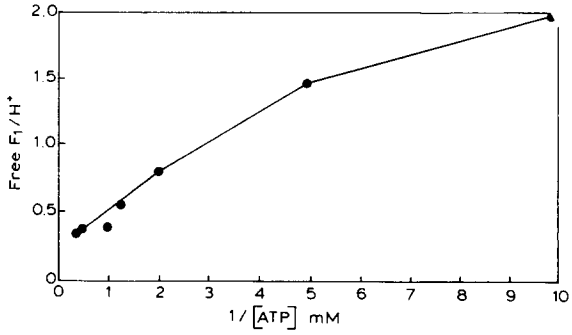

Fig. 4. Double reciprocal plot of proton released/free F_1 vs. ATP concentration. Extrapolation of the data to infinite ATP concentration indicates a maximum of three proton released per free F_1. In this experiment, free F_1 and magnesium concentrations were 47.8 nM and 5.0 mM, respectively.

showed that the proton burst is independent of Mg^{2+} concentrations and dependent on the concentration of ATP used. Without Mg^{2+} the proton burst decreased to approx. 15% of that with Mg^{2+}. The ATP hydrolysis rate increases with increasing Mg^{2+} concentration (data not shown). When free F_1 was incubated with magnesium before mixing with MgATP the proton burst did not change (data not shown). This data showed that maximum proton release from free F_1 is obtained with a metal nucleotide complex.

The ATP hydrolysis activity of bovine heart mitochondrial ATPase is decreased by modification with NBD-Cl. This inactivation results from the modification of one tyrosine per enzyme molecule [16–18]. Since this tyrosine (inactivated by NBD-Cl) is necessary for hydrolytic activity of F_1, observation of the transient kinetic proton burst upon modification with NBD-Cl was examined. The specific activity of free F_1 was determined at increasing time intervals after inactivation began. Proton released per free F_1 decreased with increasing inactivation. Fig. 5 is a plot of specific activity (as free F_1 is inactivated in time with NBD-Cl) vs. protons/free F_1 released. When free F_1 is inactivated by NBD-Cl, a decrease in the amount of protons released per free F_1 is observed. When the specific activity of free F_1 falls below 4.0 μmol/min per mg, no proton release is observed. This observation suggests that the protons are released from the catalytic site(s) or from a secondary site which depends upon the modified tyrosine to induce or transmit a conformational change. Atten-

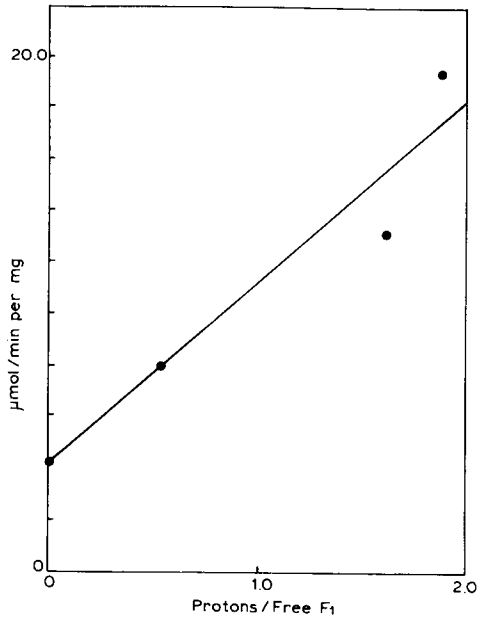

Fig. 5. A plot of the specific activity vs. protons released per free F_1 determined from an experiment in which free F_1 was inactivated by NBD-Cl. In this experiment, the concentration of free F_1, ATP and Mg^{2+} were 47.3 nM, 1.0 mM and 5.0 mM, respectively. NBD-Cl to inactivate free F_1 was 50.0 μM. Aliquots of the NBD-Cl-free F_1 inactivation reaction were taken and the inactivation stopped, so that a pre-steady-state experiment could be performed.

tion to the observation shown in Fig. 5, is necessary for understanding the transient process which induces the release of protons. It appears that the tyrosine on F_1 which is modified by NBD-Cl [16–18] is necessary in the mechanism in which free F_1 releases protons.

Tamura and Wang [11] have shown that the removal of tightly bound nucleotides from F_1-ATPase changes its reactivity toward NBD-Cl. They report inhibition by NBD-Cl and subsequent intramolecular transfer of NBD to an amino group. Our data show that complete elimination of the proton burst is observed upon modification with NBD-Cl. Therefore the data presented are consistent with the suggestion that upon NBD-Cl modification the enzyme undergoes a conformational change in which protons are not released. It must be noted that the decrease in proton release with increasing modified free F_1 can be attributed to the prevention of nucleotides binding to the enzyme.

Discussion

Previous studies of the pre-steady-state kinetics of F_1-ATPase have shown a lag period when hydrolyzing ATP [2–5]. When the enzyme is repeatedly precipitated to remove loosely bound nucleotides, an initial burst of protons is observed. A similar proton burst has also been observed with skeletal muscle Myosin ATPase [19–21]. It was reported that the proton release accompanied the binding of ATP to myosin. Bagshaw and Trentham [20] and Koretz and Taylor [21] suggested that the release of H^+ was related to the binding of ATP rather than the subsequent hydrolytic step. The proton burst reported in this paper on bovine heart mitochondrial ATPase is similar to the proton burst observed with myosin ATPase.

The cause of the proton burst observed in the transient kinetics of free F_1 can be ascertained by a qualitative review of the results. The burst is apparent only when the enzyme has been depleted of nucleotides by repeated ammonium sulfate precipitation. No proton burst is observed with native F_1. Since the proton burst is only apparent when the enzyme is repeatedly precipitated with ammonium sulfate, the enzyme must be in a slightly different conformation than native F_1 so as to allow observation of this phenomenon. The finding that Ado*PP*[NH]*P* and ADP (data not shown) also cause proton release with F_1 free of loosely bound nucleotides suggests that proton release is probably due to binding.

The results obtained when Mg^{2+} was varied while ATP and free F_1 concentrations were constant suggest that a magnesium-nucleotide complex is necessary for maximum release of protons. The finding that the binding of MgATP release a greater number of protons per free F_1 than does MgAdo*PP*[NH]*P* indicates the possibility of different nucleotide binding sites exhibiting different effects, while substrate or analogs bind. This observation suggests that a metal-hydrolyzable nucleotide complex is necessary for maximum proton release. Since it has been reported that Ado*PP*[NH]*P* binds to the regulatory sites [22] of F_1, it can be postulated that the proton burst was related to the binding of metal-nucleotide complex to the regulatory sites which released protons from the catalytic sites. The fact that a maximum of one

proton per free F_1 with MgAdo*PP*[NH]*P*, compared to three protons/free F_1 with MgATP were released suggests that the adenine nucleotide binding site on F_1 can distinguish between the two molecules. This was also suggested previously by Penefsky [15]. That is, even though Ado*PP*[NH]*P* has a remarkable similarity to ATP, the F_1 can differentiate between the two molecules. The differences observed using MgATP vs. MgAdo*PP*[NH]*P* is also seen in the concentration range needed for maximum proton release. Maximum proton release per free F_1 is observed with nM Ado*PP*[NH]*P* compared to mM ATP concentrations. This agrees with the data presented by Cross and Nalin [23] which shows Ado*PP*[NH]*P* having a high affinity binding site ($K_d = 18$ nM) on F_1. After Ado*PP*[NH]*P* binds catalysis is inhibited [23].

The fact that a maximum of three protons are released upon binding MgATP to free F_1 may support the alternating site mechanism presented by Kayalar et al. [24]. That is, under the conditions of these experiments proton release from hydrolysis is not observed until ATP binding at another catalytic site is accomplished. The negative cooperativity in proton release from free F_1 (Fig. 4) is also consistent with the alternating site mechanism indicating a change in K_d for each of the catalytic sites upon ligand binding. Since Ado*PP*[NH]*P* results in the maximum production of 1 mole protons per mole of free F_1, it is difficult to envisage cooperativity in multisite binding. The fact that Ado*PP*[NH]*P* only releases one proton/free F_1 compliments the findings of Cross and Nalin [23] who show that the binding of a single mol Ado*PP*[NH]*P*/mol of F_1 is sufficient to inhibit catalysis. This may indicate that these ligands also bind to the catalytic site but with less efficiency than MgATP (the true substrate).

In conclusion, F_1 depleted of loose nucleotides when mixed with ligands change the absorbance of phenol red which corresponds to a proton burst. This proton burst is shown to be due to a metal-nucleotide complex binding to free F_1. The fact that Mg-Ado*PP*[NH]*P* induces a proton burst shows that the burst is a function of binding and not a function of catalysis. The observation that MgATP gives the maximum burst suggests that the catalytic subunit is involved in the mechanism of the burst. The observation that NBD-Cl modification of free F_1 eliminated the proton burst suggests that the proton burst is partly due to binding of metal-nucleotide complex to the catalytic site and that the tyrosine modified is directly involved in the mechanism of proton release in nucleotide free F_1. These observations may also be supported by the alternating site model of Kayalar et al. [24]. These results also indicate that the kinetics of F_1 are a function of the state of the enzyme.

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