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Product-activation of *Escherichia coli* membrane-bound H^+-ATPase (F_1F_0-ATPase) connected with ϵ-subunit at alkaline pH

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Abstract

Mutant strain AN1518 or AN2387 (Gly48 → Asp in ϵ-subunit) and partial revertant strain AN2540 (Gly48 → Asp, Pro47 → Ser in ϵ-subunit) of *E. coli* were used in a kinetic study of membrane-bound H^+-ATPase. It was found that at pH 9.0 mutant strain AN1518 or AN2387 and partial revertant strain AN2540 gave a low initial rate, which increased with time until linearity was reached after 1–2 min. This phenomenon was prominent in mutant strains, but was not so obvious in wild-type AN346 of *E. coli*; this property is similar to F_1-ATPase reported by Cox [1]. The mechanism of the slow activation of membrane-bound H^+-ATPase was further investigated in this paper. The experimental results indicated that the hydrolytic rate of *E. coli* F_1F_0-ATPase that increased with time was membrane protein concentration- and pH-dependent, and that the product ADP produced during ATP hydrolysis is the factor causing the slow activation. Preincubation of the hydrolytic product ADP with a concentration comparable to that produced in the assay (20 μM) caused initial activation of ATP hydrolysis and abolished the slow activation. On the other hand, with the removal of ADP during the progress of the hydrolytic reaction it could be seen that the slow activation was abolished as well. In order to test the relationship between the ϵ-subunit and ADP involved in the slow activation, trypsin treatment was carried out on the membrane-bound H^+-ATPase of various strains. The activation observed after trypsin treatment was on the order of AN1518 > AN2540 > AN346. The activation effects of ADP and trypsin were not found to be additive. This implies that ADP acted in a similar way to trypsin, i.e., to cause removal of the ϵ-subunit. A tentative mechanism of the slow activation was proposed that ADP, a product of ATP hydrolysis, could induce conformational changes of F_1F_0 at alkaline pH 9.0, thus weakening the binding strength between the ϵ-subunit and other subunits of F_1F_0, and resulting in removal or partial removal of the ϵ-subunit. This further impaired the coupling of F_1 and F_0 in the mutant strains; as a consequence the rate of ATP hydrolysis was increased.

Key words: ATPase, H^+-; ϵ-Subunit; Product activation; (*E. coli*)

1. Introduction

The membrane-bound H^+-ATPase of *Escherichia coli* catalyzes the terminal step of oxidative phosphorylation: ADP + $P_i \rightleftharpoons$ ATP. The enzyme complex consists of two parts, the water-soluble F_1 portion which contains a catalytic center, and the membrane-bound F_0 portion which forms a proton pore. The F_1 portion comprises five subunits (α, β, γ, δ, ϵ) and the F_0 portion three subunits (a, b, c). The subunit structure, the amino acid sequence of the ϵ-subunit, the important amino acid residues for catalysis and subunit-subunit interactions of H^+-ATPase have been reviewed recently [2]. Isolated ϵ and γ subunits combine to form γ,ϵ complex with high affinity in vitro and are probably associated in intact F_1 [3]. The ϵ subunit of *E. coli* F_1 has been purified and characterized as a globular protein with molecular weight 15000–16000 [4]. The ϵ subunit inhibited ATP hydrolytic activity of F_1; no inhibitory effect was found when purified ϵ-subunit was added to the reconstitution of F_1-depleted membrane with F_1-ATPase which lacks the ϵ-subunit [4,5]. Recently Futai et al. [6] showed that the middle region between residue 23 and 48 of the ϵ-subunit may be essential for the functional enzyme H^+-ATPase. The mutants used in this study were mutated in residues 47 and 48 of the ϵ-subunit in the functional region.

It is well known that ADP, one of the products of ATP hydrolysis, appears to be the competitive inhibitor

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of ATP hydrolysis. It has been reported that ADP can cause irreversible inhibition of *Micrococcus* F_1-ATPase after incubation together for a long period [7]. Here we report a new phenomenon: hydrolytic activity of *E. coli* membrane-bound H^+-ATPase is found to be enhanced during reaction at alkaline pH, and ADP may be involved in the activation of *E. coli* membrane-bound H^+-ATPase which is relevant to the ϵ-subunit.

2. Materials and methods

Bacterial strains

E. coli strains used here (see [1]) were: AN346 (wild-type); AN1518 (uncC473 mutant (in ϵ-subunit, Gly48 → Asp)); AN2387 (mutant (the same background as AN1518, but contains a multicopy plasmid carrying the uncC473 allele)); AN2540 (partial revertant affecting the ϵ-subunit (in ϵ-subunit: Gly48 → Asp, Pro47 → Ser)). All strains were kindly provided by Prof. Frank Gibson's laboratory.

Chemicals

ϵ-ACA (ϵ-amino-n-caproic acid), PAB (*p*-aminobenzamidine), casein (acid hydrolysate), DHB (2,3-dihydroxybenzoic acid), atebrin, CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone), ADP (adenosine 5'-diphosphate), DCCD (*N,N'*-dicyclohexyl-carbodiimide), PK (pyruvate kinase), PEP (phospho*enol*pyruvate) were purchased from Sigma. NADH (β-nicotinamide adenine dinucleotide, reduced form), ATP (adenosine 5'-triphosphate) were purchased from Boehringer. Coomassie brilliant blue G was the product of Fluka. All the reagents were of highest quality available.

Media and growth of bacteria

The mineral salt minimal medium used and the additions were as described by Gibson et al. [8]. The cells for the preparation of membranes were grown in a 3000 ml flask (instead of a fermenter) for about 10 h.

Preparation of cell membranes

The preparation of membrane was according to the method described by Cox et al. [9] with some modifications. About 10 g of wet cells were homogenized in 40 ml buffer 1 (50 mM Tes (*N*-tris[hydroxymethyl] methyl-2-aminoethane-sulfonic acid), 10 mM magnesium acetate, 0.25 mM DTT(dithiothreitol), 0.25 mM EGTA (ethyleneglycol-bis[β-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid), 0.25 M sucrose, 6 mM PAB, 40 mM ϵ-ACA, 0.1 mM PMSF (phenyl-methyl sulfonyl fluoride), 5% glycerol (pH 7.5)), sonicated for a total of about 10 min in 20-s periods in an ice-salt bath to keep the temperature below 10°C, then centrifuged at 12000 rpm for 20 min. The yellow supernatant was centrifuged at 140000 × *g* for 60 min, and the red-brown pellet was washed with 10 ml buffer 2 (5 mM Tes, 0.5 mM DTT, 6 mM PAB, 40 mM ϵ-ACA 0.1 mM PMSF, 0.25 M sucrose, 5% glycerol (pH 7.5)), centrifuged again at 140000 × *g* for 60 min, the pellet was homogenized in 5 ml buffer 3 (20 mM TES, 1 mM magnesium acetate, 0.25 M sucrose, 0.5 mM DTT, 15% glycerol (pH 7.5)) and stored at −80°C.

ATPase hydrolytic activity determination

The assay buffer consisted of 50 mM Tris-HCl, 1 mM $MgCl_2$, 2.5 mM ATP (pH 9.0). Membrane samples were preincubated at 37°C for 15 min, and added to the assay buffer to a total volume of 0.3 ml. The reaction was carried out at 30°C except where otherwise noted. 0.5 ml of 2.5% SSA (sulphonylsalicylic acid) was used to stop the reaction. The solution was centrifuged at 10000 rpm for 2 min, and 0.5 ml of supernatant was used to determine inorganic phosphate released, with 1 μmol P_i released per min per mg membrane protein as specific enzyme unit.

Inorganic phosphate determination

The method used was as described by Lanzetta et al. [10], but Triton X-100 was used instead of Sterox.

Protein determination

The method used was as described by Sedmak et al. [11] with bovine albumin as standard.

Atebrin fluorescence quenching determination

The method used was as described by Cox et al. [12].

Different batches of membrane preparations differed somewhat in activity. The data in the tables or figures are from different batches of membrane preparations, but the comparisons between the cases with and without ADP or ATP preincubation are conducted using the same batch of membrane preparation, e.g., in Table 2.

3. Results

E. coli F_1F_0, just as F_1 [13], was in the equilibrium of two allomorph states; nearly all of the low activity state (L type) can be converted to the high activity state (H type) by incubation at 37°C for 15 min [14]. In all the following experiments, the samples were preincubated at 37°C for 15 min in buffer 3 (pH 7.5), described in Section 2, and activity was measured at 30°C or otherwise noted, because L → H type conversion was a fast process while H → L was a slow one [14].

Activation of E. coli membrane-bound H^+-ATPase during progress of reaction at alkaline pH

It can be seen from Fig. 1 that the rate of ATP hydrolysis of the H^+-ATPase of AN2540 and AN2387

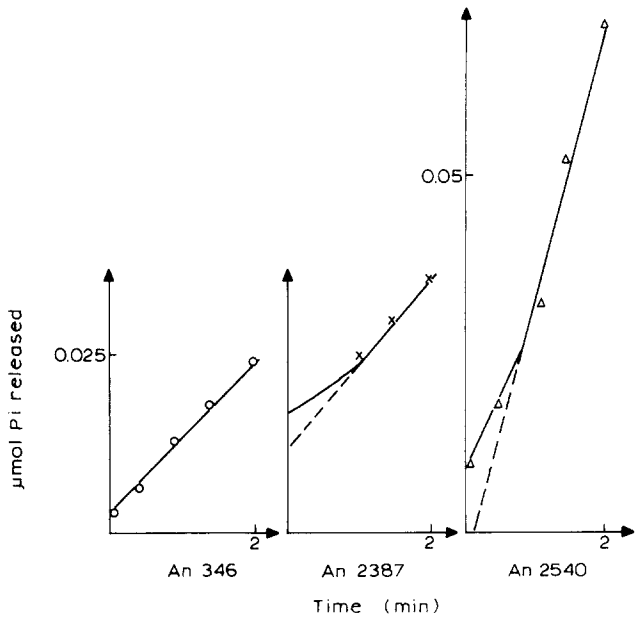

Fig. 1. Plots of P_i released during hydrolysis of ATP of membrane-bound H^+-ATPase in different strains. The method used is as described in Section 2.

were gradually increased with increase of time at pH 9.0 while in the case of wild-type strain AN346, only little activation appeared. This behavior is similar to F_1-ATPase [1]. In order to investigate pH-dependency of this activation, we measured the activity versus pH at two different incubation times (30 s and 5 min). Fig. 2 indicates that the activation in mutant strains became much more significant at higher pH.

In addition, we also determined the DCCD inhibition percentage of membrane-bound H^+-ATPase from strain AN2540 at different time intervals. It was found that the degree of inhibition decreased during the 1.5–2.0 min interval as compared with that during the 0–30 s interval at pH 9.0. The decrease in DCCD sensitivity may be possibly due to the impairment of coupling of F_1 to F_0 during the slow activation (data not shown).

Table 1
Influence of dilution of membrane from strain AN2540 on time-dependent activation (specific activity of H^+-ATPase is expressed as μmol P_i released min^{-1} mg^{-1} membrane protein)

Protein conc. (μg/ml)	18	36	180	360
Spec. act. 1 [a]	0.26	0.32	0.34	0.35
Spec. act. 2 [a]	0.60	0.60	0.47	0.36
Spec. act. 2/Spec. act. 1	2.3	1.9	1.4	1.0
Protein conc. (μg/ml)	9	18	45	90
Spec. act. 3 [a]	0.47	0.55	0.56	0.48
Spec. act. 4 [a]	0.87	0.70	0.57	0.51
Spec. act. 4/Spec. act. 3	1.8	1.3	1.0	1.06

The samples were preincubated at 37°C for 15 min, then different amounts of membrane were added to the reaction system. The activity was determined for different time intervals at 30°C (pH 9.0) as described in Section 2. [a] Spec. act. 1, 2, 3, 4 stand respectively for the average specific hydrolytic activity measured at 0–1 min, 1–2 min, 0–4 min and 4–5 min.

Influence of the membrane protein concentration on activation

It is known that ATPase activity of *E. coli* F_1-ATPase was increased when diluting the enzyme, owing to dissociating the ϵ-subunit in the presence of ATP [15]. In our case, membrane protein concentration-dependent activation also was shown (Table 1). This indicated that some dissociation process may be involved in the activation, because enzyme dilution-dependent activation is a typical property of dissociative enzymes [16].

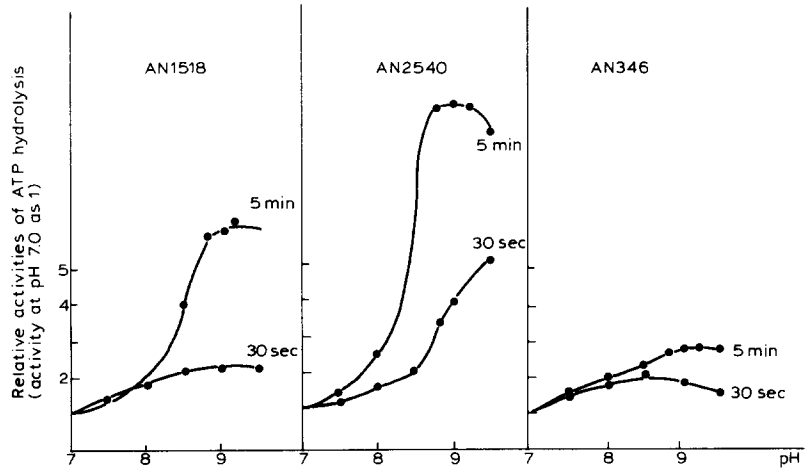

Fig. 2. Comparison of H^+-ATPase activity-pH curve between two times from membranes of different *E. coli* strains. The method used is as described in Section 2. The upper curves are for 5 min incubation, while the lower curves are for 30 s. The specific activities at pH 7.0 (labeled as 1) were as follows. For 5 min: AN1518, 0.065; AN2540, 0.110; AN346, 0.335. For 30 s: AN1518, 0.060; AN2540, 0.089; AN346, 0.243. The protein concentrations used were, for 5 min: AN1518, 125 μg/ml; AN2540, 40 μg/ml; AN346, 48 μg/ml. For 30 s: AN1518, 0.83 mg/ml; AN2540, 0.24 mg/ml; AN346, 0.32 mg/ml.

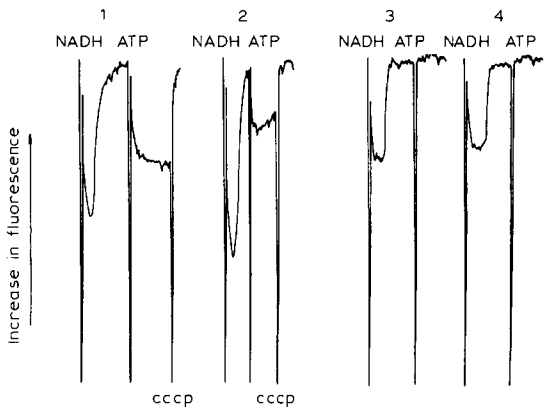

Fig. 3. Influence of trypsin treatment on atebrin fluorescence quenching of membranes. From left to right: (1) AN346, control; (2) AN346, trypsin treatment; (3) AN1518, control; (4) AN1518, trypsin treatment. The method used is as described in Section 2.

Effect of ADP and trypsin on membrane-bound F_1F_0-ATPase

Since the activation occurred during the progress of hydrolysis of substrate, the factor for activation must exist in the reaction system. Many constituents including ATP were shown to have no activating effect, but the ADP had such an effect (Table 2). In our experiment, membrane samples (10–15 mg/ml) were preincubated with 0.4 mM ADP at pH 7.5, then assayed for ATPase activity at 30°C, pH 7.5 or pH 9.0 for 1 min respectively with the same concentration of ADP added after preincubation as controls. It can be seen from Table 2 that the activation of AN346 was only about 10%, while activation of AN2540 can be as large as 50%. When preincubation was at pH 9.0, The activation became stronger (AN346 = 36%, AN2540 = 97%).

These results indicated that ADP preincubation had activation effects, i.e., the activity of membrane after

Table 2
Effect of preincubation of membrane-bound F_1F_0-ATPase with ADP or ATP or trypsin on activity (specific activity: μmol P_i released min^{-1} mg^{-1} membrane protein)

Bacterial strain	Preincubation with				
	+ATP	+ADP	+Trypsin	+Trypsin +ADP	+ADP (pH 9.0)
w.t. (AN346)	100%	110%	143%	145%	136%
AN2540	100%	150%	210%	218%	197%
AN1518	n.d.	n.d.	400%	400%	n.d.

Values are expressed relative to values for the untreated membranes for each strain. All treatments at pH 7.5 unless indicated. The amounts of protein used in each assay buffer were 80 μg/ml for AN346 and AN2540 or 280 μg/ml for AN1518.

ADP-preincubation was higher than that without ADP-preincubation, and the alkaline pH was an important condition for the exhibition of high activation effect.

So far, the results indicated that ADP could be relevant to the activation of the *E. coli* membrane-bound H^+-ATPase, with concurred lowering in its coupling efficiency. Since the effect of ADP is different on different strains which differ only in the nature of their ϵ-subunits, so the ϵ-subunit may be involved in the activation. To test this, the membrane samples were preincubated with trypsin (0.1 mg/mg membrane protein) at pH 7.5, 37°C for 15 min, then assayed for ATP hydrolysis at 30°C (pH 9.0) for 1 min. It has been reported that treatment with trypsin stimulates ATP hydrolytic activities of *E. coli* membrane-bound H^+-ATPase [17] and F_1-ATPase [18]. For the F_1-ATPase, this occurs probably owing to the removal of the ϵ-subunit [18]. In our experiment, as can be seen from Table 2, the activation by trypsin was most striking for AN1518 (mutant), and effects of ADP and trypsin were

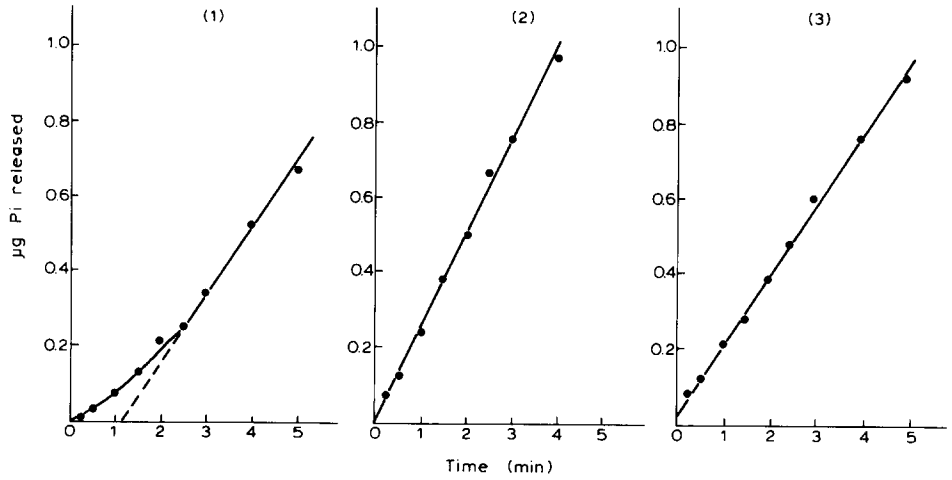

Fig. 4. Plots of P_i released during ATP hydrolysis catalyzed by AN2540 membrane-bound H^+-ATPase in the case of: (1) control; (2) preincubation with ADP at a concentration of 20 μM; (3) in the presence of ATP-regenerating system (10 mM KCl, 1 mM PEP, 10 units PK). The basic reaction media were 50 mM Tris-HCl (pH 9.0), 1 mM Mg^{2+}, 2.5 mM ATP, 20 μg membrane protein. The method used is as described in Section 2.

not additive, so two kinds of activation may share some common mechanism. Furthermore, in order to examine if the increasing activation was due to detachment of F_1 from F_1F_0 complex, NADH-dependent atebrin fluorescence quenching of membrane from strain AN1518 treated with trypsin was measured (Fig. 3).

Fig. 3 shows that F_1 seemed to be still bound to F_0 in the membranes by treatment of trypsin at pH 9.0, but ATP-dependent fluorescence quenching of AN346 was weakened after trypsin treatment. In our experiments, the concentration of trypsin used was only 1/20 to 1/50 of the concentration reported in [17] and the incubation time was only 1/2 of that in [17]. The strains used only differ in the ϵ-subunit of F_1F_0-ATPase, although the membrane-bound F_1F_0-ATPase contains phospholipids and many other proteins. Normal NADH-dependent fluorescence quenching would mean that trypsin had little influence on proteins of respiratory chain under our conditions. So it was reasonable to propose that trypsin acted mainly on H^+-ATPase, especially the ϵ-subunit. This implied that the binding of the ϵ-subunit with other subunits of mutant was weaker than that of wild-type and the treatment of trypsin probably removed or destroyed some ϵ-subunit from membrane of mutant more easily than wild-type, therefore resulting in defective coupling function of F_1 and F_0 thus activating ATP hydrolysis.

Effect of removing ADP produced during the progress of ATP hydrolysis

In order to make sure that product ADP activated the ATP hydrolysis gradually during the progress of the reaction, an ATP-regenerating system was added to the assay. It could be seen that the slow activation phenomenon of the ATP hydrolysis was abolished significantly (Fig. 4), but the slope of curve 3 in Fig. 4 is somewhat higher than the initial rate without regenerating system. We have done additional experiments to find out the causes. It was reported [19] that K^+ activates ATPase from *Alcaligenes faecalis* and bovine heart F_1. Here we also found that the higher slope of curve 3 was caused by 10 mM KCl in the regenerating system (PK in the regenerating system needs monovalent cation as activator, so KCl was used). On the other hand, when a suitable concentration of ADP (20 μM) comparable to that produced in the beginning of the linear rate in the assay was preincubated with the membrane, it caused the abolishment of slow activation also (Fig. 4). These results mentioned above lead us to propose a tentative mechanism of ADP activation that ADP, a product of ATP hydrolysis in the progress of the reaction, induces the conformational changes of H^+-ATPase in the alkaline pH, thus weakening the binding strength between the ϵ-subunit and other subunits of H^+-ATPase. This results in the dissociating of the ϵ-subunit, so the removal of it or of a part of it causes further impairment (in the case of mutant) of F_1, F_0 binding and coupling. As a consequence, the ATP hydrolysis is increased.

4. Discussion

Our experiments showed that the observed activation phenomenon during the hydrolytic reaction of H^+-ATPase at higher pH had an intimate connection with a dissociation process, and this dissociation weakened the coupling between F_1 and F_0. This process could be easily observed in our experiments possibly because the concentrations of membrane protein used in our assay system were lower than in other methods, and the sensitivity of inorganic phosphate determination used was about ten times higher than other methods.

The different results in different strains which differed only in their ϵ-subunits lead us to a conclusion that the ϵ-subunit is possibly involved in the phenomena. The role played by the ϵ-subunit is binding and coupling of F_1 to F_0 [6,14]. It is known that the ϵ-subunit in F_1F_0-ATPase may not have intrinsic inhibitory activity [4]. Trypsin treatment in our experiments showed that apparently the normal strain AN346 (wild type) only exhibited little activation, while the mutant significantly increased in activation. In mutants, the binding of ϵ-subunit is already defective or weakened, so that at high pH this subunit is more sensitive to trypsin and so is more easily dissociated from F_1. Removal of the ϵ-subunit or part of it could lead membrane-bound H^+-ATPase into a defective coupling state and lead to an increase in ATPase activity and a decrease in DCCD sensitivity. So the activation phenomena were apparent in the strains AN1518 and AN2540 with altered ϵ-subunit.

It is well known that F_1 of *E. coli* has several nucleotide binding sites, some of which can bind ADP, but their functions are still obscure. Laget and Smith have reported that ATP and ADP influenced the dissociation of the ϵ-subunit from the purified *E. coli* F_1-ATPase [15]. Our experiments showed that incubation with ADP in alkaline pH could activate membrane H^+-ATPase and lower its coupling efficiency. We also found that trypsin treatment had similar effects, but effects of ADP and trypsin were not additive. This implied that ADP in alkaline pH acted in a similar way to trypsin. These results suggested that in alkaline pH ADP could induce some conformational change of *E. coli* membrane-bound H^+-ATPase. In this new conformation, the binding between the ϵ-subunit and other subunits of F_1F_0 could become loosened, and the dissociation of the ϵ-subunit may be accelerated. The mechanism of the activation phenomena during the reaction may be considered as the same activation

mechanism caused by ADP preincubation in alkaline pH. ADP preincubation increased the initial rate of ATP hydrolysis, however, slow activation did not occur again since the concentration of ADP used in preincubation was in the range of possible production of ADP during the reaction. Furthermore, removing ADP by adding ATP-regenerating system in the assay could significantly abolish the activation phenomenon during progress of the reaction.

Although ATPase activity and coupling function of the revertant strain AN2540 are restored partially, the activation during reaction at alkaline pH for membrane of AN2540 appeared stronger than that of membrane from the mutant strains AN2387 or AN1518 (Figs. 1, 2). This may imply that in the revertant strain AN2540, the substitution of Pro47 by Ser in the ϵ-subunit caused partial restoration of the local conformation needed for normal ATPase activity and coupling function, as suggested by Cox et al. [1], but other conformational changes induced by revertant strain with two amino acid mutations may be responsible for activation at alkaline pH and ADP effects during reaction. Amino acid residue substitutions of the mutants used in this study are located in the essentially functional region of the ϵ-subunit [6], and as this phenomenon was observed at alkaline pH, the physiological significance of this activation is not clear.

5. Acknowledgements

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