Vacuolar H\(^+\)-ATPase (V-ATPase) consists of a catalytic head, a stalk part and a membrane domain. We indirectly investigated the interaction between the A subunit (catalytic head) and the E subunit (stalk part) using an ATP analogue, adenosine 5\(^\prime\)-[\(\beta,\gamma\)-imino]triphosphate (AMP-PNP), which holds the enzyme in the substrate-binding state. AMP-PNP treatment caused a mobility shift of the E subunit with a faster migration in SDS/polyacrylamide gel electrophoresis without a reductant, while ATP treatment did not. A mobility shift of the E subunit has been detected in several plants. As polypeptides with intramolecular disulfide bonds migrate faster than those without disulfide bonds, the mobility shift may be due to the formation of an intramolecular disulfide bond by two cysteine residues conserved among several plant species. The mobility shift may be involved in the binding of AMP-PNP to the ATP-binding site, which exists in the A and B subunits, as it was inhibited by the addition of ATP. Pretreatment with 2\(^\prime\)-3\(^\prime\)-O-(4-benzoylbenzoyl)-ATP (Bz-ATP), which modifies the ATP-binding site of the B subunit under UV illumination, did not inhibit the mobility shift of the E subunit caused by AMP-PNP treatment. The response of V-ATPase following the AMP-PNP binding may cause a conformational change in the E subunit into a form that is susceptible to oxidation of cysteine residues. This is the first demonstration of interaction between the A and E subunits in the substrate-binding state of a plant V-ATPase.

**Keywords:** vacuolar H\(^+\)-ATPase; E subunit; plant; conformational change; ATP analogue.

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Vaccular H\(^+\)-ATPase (V-ATPase) functions in the membranes of acidic compartments in eucaryotic cells and in the plasma membranes of certain animals and bacteria [1–4]. The V-ATPase complex is composed of a peripheral V\(_1\) domain responsible for ATP hydrolysis and an integral V\(_0\) domain responsible for proton translocation [5,6]. The V\(_1\) domain of a 570-kDa complex consists of a catalytic head (A\(_1\)B\(_2\) complex) and stalk part (C to H subunits). The membrane integral V\(_0\) domain of a 250-kDa complex contains at least five different subunits with molecular masses of 17–100 kDa (a to d subunits). Electron microscopic studies have indicated that the structure of V-ATPase is similar to that of F-ATPase [7–9]. High-resolutional image analysis of the bovine heart mitochondrial F\(_1\) domain is similar to that of F-ATPase [7–9]. Recent studies have demonstrated that the \(\gamma\) subunit rotation against the \(\alpha_3\beta_3\) complex in the catalytic process is driven by the energy generated by ATP hydrolysis in the F\(_1\) domain [11,12]. In the process of ATP synthesis, the electrochemical potential causes rotation of the \(\gamma\) subunit. It has been demonstrated that the electrochemical potential is transduced to energy for the \(\gamma\) subunit rotation through the interaction of a and c subunits in the F\(_3\) domain [13,14].

Although the energy-transducing mechanism of V-ATPase is thought to be similar to that of F-ATPase, it has not yet been determined which subunit of V-ATPase corresponds to the \(\gamma\) subunit of F-ATPase. As the C, D and E subunits have been demonstrated to interact with the proteolipid subunits (c or c') [15], these subunits might play a potential role in transmitting the energy of ATP hydrolysis from the V\(_1\) to the V\(_0\) domain. Among these subunits, either the C [9,16], the E [17,18] or a pair of the D and E subunits [19] may be the candidate for the \(\gamma\) subunit homolog. Xie [20] reported that at least the A, B, C, E and G subunits, but not the D and F subunits, are essential for ATP hydrolysis on the bovine enzyme. On the other hand, the A, B and D subunits of the *Thermus thermophilus* enzyme formed a complex similar to the \(\alpha_3\beta_3\gamma\) complex [9].

An X-ray crystallographic study has shown that conformational change of the \(\beta\) subunit occurs due to the binding of adenosine 5\(^\prime\)-[\(\beta,\gamma\)-imino]triphosphate (AMP-PNP) [10]. It is thought that this conformational change is transmitted to rotating energy of the subunit during ATP hydrolysis [21,22]. Because the A subunit of V-ATPase corresponds to the \(\beta\) subunit of F-ATPase, the binding of AMP-PNP may cause conformational change in the A subunit prior to the rotation of stalk subunits. A previous study using two-dimensional electrophoresis and immunoblotting has demonstrated that the mung bean V-ATPase...
contains at least three E subunit isoforms [23]. In the present study, we found that the electrophoretic mobility of the E and 51-kDa subunits in SDS/PAGE analysis without a reductant shifted to apparent lower sizes when mung bean V-ATPase was treated with AMP-PNP. The mobility shift of the E subunit may be closely related with the conformational change of V-ATPase dependent on AMP-PNP binding. It is expected that detailed study of this phenomenon will provide new information about the interaction between the catalytic subunit A and stalk subunit E of V-ATPase. The present study has provided indirect evidence of interaction between the A and E subunits in the substrate-binding state of a plant V-ATPase. This conformational change during ATP hydrolysis is discussed in this paper.

MATERIALS AND METHODS

Materials

Seeds of the mung bean (Vigna radiata L. cv. Wilczek) were imbibed with 1 mm CaSO₄ and germinated in the dark at 26 °C for 3.5 days. Lyso phosphatidylcholine (egg yolk, type I), phosphatidylcholine (soybean, type IV-S) and 2'-3'-O-(4-benzoylbenzoyl)-ATP (Bz-ATP) were purchased from Sigma. ATP and AMP-PNP were obtained from Roche Molecular Biochemicals. QAE-Toyopearl 550C was obtained from Tosoh (Japan). Other products used were of analytical grade.

Purification of V-ATPase

Vacuolar membranes were prepared from tissue homogenate by differential and floating centrifugation as previously reported [24,25]. The vacuolar membranes were treated with 5% (w/v) Triton X-100 in 0.1 M KCl prior to solubilization of V-ATPase with 2 mg·mL⁻¹ lyso phosphatidylcholine, and then V-ATPase was purified by chromatography on a QAE-Toyopearl column [24].

AMP-PNP, ATP and Bz-ATP treatment

The solvent of purified enzyme was exchanged with TG buffer [20 mm Tris/acetate buffer (pH 7.2) and 20% (w/v) glycerol] containing 0.1% (w/v) Triton X-100 at 0 °C using an Ultrafree filter unit (UFC3 LTK 00, Millipore). Chemical treatment of ATPase was usually performed at 30 °C. Finally, the samples (0.2 μg·μL⁻¹) were mixed with TG buffer containing 1 μg·μL⁻¹ phosphatidylcholine, 50 mm NaCl, 0.01% (w/v) Triton X-100, AMP-PNP or ATP of an appropriate concentration in each experiment and the same concentration of MgSO₄. After treatment with AMP-PNP or ATP, N-ethylmaleimide was added to the mixture to a final concentration of 5 mM for alkylation of cysteine residues, and then the mixture was incubated at 30 °C for 10 min. The Bz-ATP treatment was perfomed by the same method as that used for the AMP-PNP treatment, except for UV illumination for 10 min at 0 °C. After the Bz-ATP treatment, free Bz-ATP was removed from the mixture with an Ultrafree filter unit (UFC3 LTK 00).

Analytical measurements

Protein samples were treated with Laemmli’s sample buffer without a reducing reagent for 30 min. SDS/PAGE on 13% polyacrylamide gel was carried out by the method of Laemmli [26]. Proteins in the 2D PAGE gels were visualized with silver-staining [27]. Antibodies against the pea E subunit isoforms of 40 and 37 kDa were individually prepared as described previously [23]. A mixture of the two antibodies was designated as anti-E. Immunoblotting with anti-E was carried out by the standard method [28]. The antigens on poly(vinylidene difluoride) (PVDF) membranes were visualized with alkaline phosphatase-conjugated goat anti-(rabbit IgG) IgG using 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitroblue tetrazolium as color development reagents [29]. The protein concentration was determined by the method of Bradford using γ-globulin as a standard [30]. The ATP hydrolysis activity was assayed at 30 °C for 30 min in 0.25-mL of medium containing 3 mM ATP, 3 mM MgSO₄, 50 mM KCl, 1 mM sodium molybdate, 20 μg of phosphatidylcholine (soybean,
RESULTS

Mobility shift of V-ATPase subunits in SDS/PAGE analysis without a reductant by treatment of the enzyme with AMP-PNP

Mung bean V-ATPase consists of 10 subunits: A (68 kDa), B (57 kDa), D (32 and 29 kDa), E (38 and 37 kDa), c (16 kDa), and 100-kDa, 51-kDa, 44-kDa, 13-kDa and 12-kDa subunits [23]. There are isoforms of D and E subunits in pea and mung bean V-ATPases. In mung bean V-ATPase, three isoforms that consist of one 38-kDa protein with pI 5.9 and two 37-kDa proteins with pI 6.3 and pI 6.5 have been characterized as the E subunit and are collectively designated as the E subunit in this report. Abrahams et al. [10] demonstrated that conformational change in the β subunit could be achieved by treatment of F-ATPase with a synthetic ATP analogue, AMP-PNP. This report led us to examine which subunit of plant V-ATPase changes its conformation. AMP-PNP is a synthetic ATP analogue that inhibits the activities of ATP-dependent enzymes. As the reagent is not hydrolyzed by ATPase, the enzyme bound with AMP-PNP is maintained in the substrate (ATP)-binding state.

After the V-ATPase purified from mung bean hypocotyls had been treated with 3 mM AMP-PNP, the conformational change in each subunit was examined by diagonal electrophoresis under a nonreducing condition in the first dimension and under a reducing condition in the second dimension (Fig. 1). The results of the diagonal electrophoresis study revealed that the mobility of 38- and 37-kDa

![Fig. 2. Effect of N-ethylmaleimide (NEM) treatment after AMP-PNP treatment.](image)

![Fig. 3. Effects of reaction time and AMP-PNP concentration on the mobility shift of the E subunit.](image)
spots (E subunit isoforms) as usually detected in the presence of 2-mercaptoethanol shifted to apparent lower sizes of 30 and 29 kDa under a nonreducing condition, respectively, when V-ATPase was treated with AMP–PNP (Fig. 1B, arrowheads). Although these spots changed their mobility even in the absence of AMP–PNP (Fig. 1A), the intensities of the spots with faster electrophoretic mobility were remarkably low. The 51-kDa subunit also changed its mobility to 48, 45 and 44 kDa under a nonreducing condition (Fig. 1B, arrowheads). In general, polypeptides with intramolecular disulfide bonds migrate faster than do those without disulfide bonds [32]. Thus, it can be assumed that the mobility shift of the subunit is due to the formation of an intramolecular disulfide bond. In the present study, we further investigated the formation of an intramolecular bridge of the E subunit by immunoblotting with the specific antibodies to E subunit isoforms, anti-E.

Next, the redox state of the cysteine residues in the E subunit was examined using N-ethylmaleimide, which irreversibly alkylates cysteine residues. The purified V-ATPase was treated with AMP-PNP and then with N-ethylmaleimide to protect free cysteine residues. The N-ethylmaleimide treatment did not affect the migration of the reduced form (38 and 37 kDa) in the absence of AMP-PNP (Fig. 2A, lanes 4 and 5). As the mobility shift of the E subunit was hardly affected even by 50 mM of N-ethylmaleimide (Fig. 2A, lane 3), it was thought that mobility shift was most likely caused by AMP-PNP treatment with the enzyme, not by the addition of SDS sample buffer without 2-mercaptoethanol. On the other hand, when the N-ethylmaleimide treatment after AMP-PNP treatment was omitted, a mobility shift of the 38-kDa subunit to 29.7 and that of the 37-kDa subunit to 29.3 and 28.5 kDa were observed (Fig. 2A, lane 1 and Fig. 2B). At present, it is unclear why the apparent mobility shifts of E subunits due to AMP–PNP treatment without N-ethylmaleimide are different from those with N-ethylmaleimide. In the immunoblotting experiments, the intensities of the E subunit bands that had shifted to 30 and 29 kDa were weaker than those of the original bands of 38 and 37 kDa. This may have been caused by the differences in immunoreactivity between the two conformations.

**AMP-PNP treatment in various conditions**

Various experimental conditions were tested to understand the mobility shift of the E subunit caused by AMP-PNP treatment. First, it was revealed that the mobility shift of the E subunit was dependent on the AMP-PNP concentration and reaction time (Fig. 3). The incidence of mobility shifts increased with a rise in the AMP-PNP concentration and elapse of reaction time. Thereafter, the enzyme was treated with 3 mM AMP-PNP for 60 min at 30 °C. Next, the necessity of Mg$^{2+}$ during the AMP-PNP treatment was investigated, because ATP binds to the V-ATPase in a form of Mg-ATP. A mobility shift of the E subunit hardly occurs in the absence of Mg$^{2+}$ (Fig. 4). The mobility shift was completely prevented by chelating Mg$^{2+}$ with EDTA (Fig. 4). In order to study the effects of a redox condition on the mobility shift of the E subunits, 2-mercaptoethanol was added to the reaction medium of AMP-PNP treatment. The presence of 5 mM of 2-mercaptoethanol in the medium
strongly inhibited the E subunit mobility shift (Fig. 5A). In contrast, the mobility shift of the E subunit was not affected by the addition of 2-mercaptoethanol to a final concentration of 5 mM after the AMP-PNP treatment (Fig. 5B).

**ATP and Bz-ATP treatment**

Treatment of the V-ATPase with 3 mM ATP for 60 min at 30 °C did not bring about a mobility shift (data not shown). As mung bean V-ATPase exhibits little activity of ATP hydrolysis or H⁺ pump at 0 °C [33], it had been speculated that treatment of the enzyme with ATP at 0 °C would lead to a similar result as that in the case of AMP-PNP. A mobility shift, however, was not detected after treatment with an actual substrate ATP even at 0 °C for 60 min (Fig. 6A), while a mobility shift was partly induced by AMP-PNP treatment at 0 °C. These results suggest that an intramolecular disulfide bond of the E subunit is not formed by the addition of ATP, which can not substitute for AMP-PNP. To obtain more information, the competition between AMP-PNP and ATP for the mobility shift of the E subunit and ATP hydrolysis was examined. Coexistence of ATP at more than 1 mM inhibited the mobility shift induced by AMP-PNP (Fig. 6B), but the addition of excessive ATP after AMP-PNP treatment did not inhibit the mobility shift (Fig. 6C, lane 2). As shown in Table 1, ATP hydrolysis activity in the presence of 3 mM ATP was significantly inhibited by the addition of 1 mM AMP-PNP.

It is known that Bz-ATP covalently binds to the ATP binding site of the B subunit, not the A subunit, by irradiation of long-wavelength UV during the incubation of a reaction mixture of plant or animal V-ATPases and inhibits the enzyme activity [34,35]. Pretreatment of mung

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**Table 1. Inhibition of ATPase activity by ATP analogues.** ATPase activity was assayed at 30 °C for 30 min in medium containing 3 mM ATP as described in Materials and methods. The values of ATPase activity are mean ± SE for three independent experiments.

<table>
<thead>
<tr>
<th>Condition (concn in mM)</th>
<th>ATPase activity (μmol Pi · h⁻¹·mg protein⁻¹)</th>
<th>% Control</th>
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<tbody>
<tr>
<td>Competition by AMP-PNP</td>
<td></td>
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<tr>
<td>0</td>
<td>15.1 ± 0.4</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>3.5 ± 0.3</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>0.4 ± 0.4</td>
<td>3</td>
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<tr>
<td>Pretreatment by Bz-ATP</td>
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<tr>
<td>0</td>
<td>8.83 ± 0.45</td>
<td>100</td>
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<tr>
<td>0.1</td>
<td>0.288 ± 0.017</td>
<td>5.1</td>
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**Fig. 6. Effect of ATP treatment.** (A) The purified enzyme (4 μg) was treated with reaction buffer in the absence (lane 1) or presence of 3 mM of ATP (lane 2) or 3 mM AMP-PNP (lane 3) at 0 °C for 60 min and then treated with 5 mM N-ethylmaleimide at 0 °C for 10 s. (B) The purified enzyme (4 μg) was treated with 3 mM AMP-PNP in the presence of 0 mM (lane 1), 0.1 mM (lane 2), 1 mM (lane 3) or 3 mM (lane 4) of ATP at 30 °C for 60 min and then treated with 5 mM N-ethylmaleimide at 30 °C for 10 min (C) The purified enzyme (4 μg) was treated with 3 mM AMP-PNP at 30 °C for 120 min (lane 1), 15 mM of ATP at 30 °C for 60 min after treatment with 3 mM AMP-PNP at 30 °C for 60 min (lane 2) or 3 mM AMP-PNP in the presence of 15 mM of ATP at 30 °C for 120 min (lane 3), followed by treatment with 5 mM N-ethylmaleimide at 30 °C for 10 min. SDS/PAGE and immunoblotting with anti-E were performed to detect the E subunit. The arrowheads show the E subunits.

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**Fig. 7. Effect of Bz-ATP treatment.** The purified enzyme (4 μg) was treated with 0 mM (lanes 1 and 2) or 100 mM (lanes 3 and 4) of Bz-ATP with UV illumination for 10 min at 0 °C. After the Bz-ATP treatment, the Bz-ATP was removed from the mixture as described in Materials and methods. Each sample was then treated without (lanes 1 and 3) or with (lanes 2 and 4) 3 mM AMP-PNP at 30 °C for 60 min, followed by treatment with 5 mM N-ethylmaleimide at 30 °C for 10 min. SDS/PAGE and immunoblotting with anti-E were performed as described in the legend of Fig. 2. The arrowheads show the E subunits.
bean V-ATPase with Bz-ATP clearly inhibited the ATP hydrolysis (Table 1). A mobility shift of the E subunit was not induced by Bz-ATP, and the addition of AMP-PNP to a Bz-ATP-treated sample caused a mobility shift (Fig. 7).

**DISCUSSION**

Conformational change in the E subunit

This study demonstrated that the mobility shift of the E subunit to an apparent lower size in SDS/PAGE analysis without a reductant is caused by the AMP-PNP treatment of V-ATPase. As no protein spots that interacted with the E subunit were detected by analysis of diagonal electrophoresis (Fig. 1), it is thought that the mobility shift is due to formation of an intramolecular disulfide bond in each polypeptide. The intramolecular disulfide bond formation may be due to conformational change in the E subunit into a form that is susceptible to oxidation of cysteine residues in a reaction medium. As reported previously, mung bean V-ATPase has three isoforms of the E subunit, one of 38 kDa and two of 37 kDa, and a single V-ATPase complex may contain either molecule of these isoforms [23]. After AMP-PNP treatment of V-ATPase, the mobility of E subunit was detected by analysis of diagonal electrophoresis (Fig. 1), it is thought that the mobility shift is due to formation of an intramolecular disulfide bond in each polypeptide. This result was observed after activation of AMP-PNP but not ATP (Fig. 6A), Bz-ATP (Fig. 7, lane 3) or ADP (data not shown).

As the coexistence of ATP and AMP-PNP in a reaction medium inhibited the mobility shift of E subunit caused by AMP-PNP treatment (Fig. 6B), the binding of AMP-PNP to the ATP-binding site obviously results in a conformational change in the E subunit. Moreover, a mixture of 3 mM ATP and AMP-PNP inhibited both the mobility shift of the E subunit and ATP hydrolysis activity. There are two possible reasons for this phenomenon: (a) the AMP-PNP binding site, which is involved in the mobility shift, is not the same as the ATP-binding site for ATP hydrolysis, or (b) it is the same, but neither ATP nor AMP-PNP binds to the ATP-binding site in the case of coexistence. It has been reported that the ATP-binding site exists in the A and B subunits [5]. The cDNAs for the C to H subunits have been cloned for *Arabidopsis thaliana* (relevant references: C, NCB accession no. AF208261; D, [36]; E, [37]; F, NCB accession no. T01087; G, NCB accession no. AAF24609; H, NCB accession no. AL356013), of which subunit E also causes a mobility shift dependent on AMP-PNP treatment (data not shown). The result of a motif search (http://www.motif.genome.ad.jp/) did not reveal any ATP-binding sites in these subunits. Treatment with Bz-ATP did not inhibit the mobility shift of the E subunit dependent on AMP-PNP treatment (Fig. 7, lane 4), suggesting that the B subunit was not involved in the mobility shift of the E subunit. Finally, it is concluded that the binding of AMP-PNP to the ATP-binding site of the A subunit leads to conformational change in the E subunit. This is important evidence of interaction between V-ATPase subunits in the process of ATP hydrolysis.

In the case of F-ATPase, it has been demonstrated that the C-terminal domain of the β subunit moves like a hinge when AMP-PNP binds to the β subunit [10]. This conformational change causes rotation of the stalk subunits during ATP hydrolysis [21,22]. The β subunit of F-ATPase corresponds to the subunit of V-ATPase. Therefore, the binding of AMP-PNP to the A subunit may lead to conformational change in the C-terminal domain of the A subunit. The D or E subunit may interact with the Aβ₁ complex, because none of the deletion mutants of the subunit genes in yeast were able to form an Aβ₁ complex [19]. In the *Manduca sexta* enzyme, an A−E−F complex is generated by the addition of the cross-linker reagent CuCl₂ in the presence of Ca-ADP [18]. In our preliminary experiments with a cross-linking reagent, the E subunit of mung bean enzyme was found to be adjacent to the B subunit (Y. Kawamura, K. Hotsubo, K. Arakawa & S. Yoshida, unpublished results). It is therefore very probable that conformational change in the Aβ₁ complex influences the E subunit conformation, although the precise position between the stalk subunits in the V₁ domain is unknown. Recently, Gruber *et al.* [18] have reported that the *M. sexta* E subunit causes relative positional changes depending on the nucleotide. Their results support the hypothesis that conformational change in the E subunit is caused by conformational change in the A subunit resulting from AMP-PNP treatment, although its detailed mechanism is unknown. The process of ATP hydrolysis in V-ATPase is assumed to involve at least four steps: (a) binding of Mg-ATP and H₂O, (b) conformational change in the enzyme by the
binding of Mg-ATP, (c) a state after ATP hydrolysis, and (d) release of P_i followed by Mg-ADP [38]. The conformational change in the E subunit due to AMP-PNP treatment may occur in step b, because the AMP-PNP is not hydrolyzed. As the V-ATPase of mung bean hardly hydrolyzed ATP at 0 °C [33], it is thought that the ATP-binding state of the enzyme is maintained. ATP treatment at 0 °C, however, did not induce a mobility shift in the E subunit (Fig. 6A). One possible reason for this is that ATP bound to the catalytic subunits is quickly hydrolyzed to ADP and P_i, but these products are hardly released from these subunits at 0 °C and, consequently, it may be difficult for the enzyme to be maintained in the ATP-binding state. In the process of ATP hydrolysis, step c may follow step b instantaneously, and the lifetime of the oxidizable state of the E subunit may therefore be too short for attack by the oxidant. However, the present study has provided no findings that support this hypothesis.

Cysteine residues of the intramolecular disulfide bond in the E subunit

When the purified V-ATPase from the pea and the crude membrane fraction from A. thaliana were analyzed by immunoblotting, a mobility shift of the E subunit caused by AMP-PNP was observed (data not shown). Thus, conformational change in the E subunit might be common in plant V-ATPases. The cDNAs for the E subunit have been cloned for five plants, including A. thaliana [37,39–41]. All of the sequences possess two conserved cysteine residues, such as Cys134 and Cys185 in the A. thaliana subunit (Fig. 8). However, these cysteine residues are not conserved in the E subunit of other organisms. Thus, the disulfide bond formation in the E subunit may be unique to plant V-ATPases.

A question arises concerning the nature of the oxidant for the conformational change of the E subunit. It is likely that oxygen dissolved in the reaction medium of AMP-PNP treatment would act as an oxidant for cysteine residues of the E subunit in the assay. The results shown in Fig. 5A indicate that 2-mercaptoethanol added to the AMP-PNP solution indirectly inhibited the formation of a disulfide bond in the E subunit, as the addition of 2-mercaptoethanol after the AMP-PNP treatment did not result in the inhibition of the formation of a disulfide bond (Fig. 5B). Therefore, the inhibition of disulfide bond formation of the E subunit by 2-mercaptoethanol in the reaction medium of AMP-PNP treatment may be due to the reduction of the oxidant, probably dissolved oxygen, in the medium by 2-mercaptoethanol, not due to direct interaction with cysteine residues of the E subunit (Fig. 5A). In a preliminary experiment, the effects of oxidative conditions on the conformational change in the E subunit were investigated. Incubation of V-ATPase at 37 °C for 24 h without a reductant caused a partial mobility shift of the E subunit, and treatment of the enzyme with 20 μM CuCl_2 induced a complete mobility shift as did the AMP-PNP treatment (data not shown). These results suggest that an oxidative condition may promote intramolecular disulfide bond formation in the E subunit. However, treatment with the oxidative form of glutathione (10 mM), which inhibited the ATPase activity by about 20%, did not cause a mobility shift of the E subunit (data not shown). It is possible that the oxidant that caused the formation of a disulfide bond in the E subunit is either a smaller molecule or a stronger oxidant than the oxidative form of glutathione, or that some mediators are necessary for oxidation by glutathione in this condition. Although identification of the oxidants in the reaction medium or plant cell is necessary to get more insights into the mechanism of intramolecular disulfide bond formation in the E subunit, it is possible that the formation of an intramolecular disulfide bond in the E subunit may be influenced by the redox condition in the plant cell.

We have not described the relationship between the intramolecular disulfide bond formation of the E subunit and the reaction mechanism of V-ATPase in this report. However, several studies have shown that the V-ATPases of plants [42], animals [43] and yeast [44] are regulated by the redox state and that the oxidation of V-ATPase causes dysfunction of the enzyme. Dschida and Bowman [45] assumed that the stalk domain of the Neurospora crassa V-ATPase is regulated by the redox state, although it is unknown which subunits in this domain are regulated. In the case of chloroplast ATP synthase, the activity is distinctly regulated through the redox state of the cysteine residues of the subunit [46,47]. Thus, it is speculated that the intramolecular disulfide bond formation of the E subunit is related to the redox regulation of plant V-ATPase.

As shown in Fig. 1B, the mobility shift of the 51-kDa subunit simultaneously appeared as three spots, and a part of the 51-kDa subunit did not shift. This result implies that the spot of 51 kDa may contain some isoforms. In our previous study, the 51-kDa subunit of the mung bean was estimated to correspond to the yeast H subunit [23]. It has been reported that there is communication between the A and H subunits in bovine [48] and yeast [49] enzymes and that two H subunit isoforms were present per one enzyme complex of bovine [48]. The communication between the A subunit and the 51-kDa subunit remains to be determined at the molecular level.

This report is the first to describe observation of a redox change in the E subunit through the A subunit in a substrate-binding state. Further study is needed to elucidate the coupling mechanism between ATP hydrolysis and E subunit conformational change, and the mechanism of the redox reaction in the E subunit.

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