Inhibition of tonoplast ATPase from etiolated mung bean seedlings by fluorescein 5'-isothiocyanate

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Fluorescein 5'-isothiocyanate (FITC) was used to modify the lysine residue in the active site of tonoplast H⁺-ATPase from etiolated mung-bean (*Vigna radiata* L.) seedlings. FITC caused marked inactivation of the enzyme activities of both membrane-bound and soluble ATPase and its associated H⁺ translocation. The SDS/PAGE pattern revealed that the FITC-binding site was in the large (A) subunit of ATPase. Inhibition could be substantially prevented by its physiological substrate ATP, pyrophosphate and nucleotides in the decreasing order: ATP > pyrophosphate > ADP > AMP > GTP > CTP > UTP. The mode of inhibition by FITC was competitive with respect to ATP. Loss of ATPase activity followed pseudo-first-order kinetics with a *K_i* of 0.33 mM, a minimum inactivation half-time of 110 s, and a first-order rate constant of 0.244 s⁻¹. A double-logarithmic plot of apparent rate constant versus FITC concentration gave a slope of 0.913, indicating that inactivation results from reaction of at least one lysine residue at the catalytic site of the large subunit. Labelling studies indicated that the incorporation of approx. 1 mol of FITC/mol of ATPase is sufficient to inhibit ATPase completely. The enhancement and blue shift of emission maxima of FITC after modification of ATPase indicated that the labelled lysine residue was located in a relatively hydrophobic domain.

INTRODUCTION

The tonoplast of higher plants contains an electrogenic proton-translocating ATPase (Sze, 1985; Wang *et al.*, 1989) which acidifies the interior of vacuoles and generates a proton-motive force for various secondary active transport systems of solutes across the membrane. This proton pumping is characteristically inhibited by *N*-ethylmaleimide (NEM), NO₃⁻, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl) and *NN'*-dicyclohexylcarbodi-imide (DCCD), but can be stimulated by Cl⁻ (Sze, 1985; Wang & Sze, 1985; Wang *et al.*, 1989).

The vacuolar H⁺-ATPase of higher plants is a multimeric enzyme of molecular mass 400–600 kDa consisting of seven to nine major polypeptides (Parry *et al.*, 1989; Matsuura-Endo *et al.*, 1990). The subunits with molecular masses of about 67–70 (A-subunit), 52–60 (B-subunit) and 16 (DCCD-binding 16 kDa proteolipid subunit) kDa are essential to ATPase activity (Bowman *et al.*, 1986; Mandala & Taiz, 1986; Manolson *et al.*, 1985; Randall & Sze, 1986). The 70 kDa (A) subunit is implicated as the active site of ATP hydrolysis (Randall & Sze, 1986; Bowman *et al.*, 1986). The 58 kDa (B) subunit may play a regulatory role (Bowman *et al.*, 1986; Manolson *et al.*, 1985). The 16 kDa (DCCD-binding proteolipid) subunit can bind DCCD and is probably involved in proton translocation (Mandala & Taiz, 1986; Manolson *et al.*, 1985; Randall & Sze, 1987).

The A-subunit of carrot (Zimnak *et al.*, 1988) and both the A- and B-subunits of *Neurospora crassa* (Bowman *et al.*, 1988a,b) vacuolar ATPase have been sequenced. The A-subunits are highly conserved and are related to the F-type ATPase β -subunits (Nelson, 1989). A fragment of amino acid sequence in the A-subunit of carrot tonoplast ATPase is homologous with a region of the β -subunit of *Escherichia coli* F₁-ATPase, which is believed to contain the universal nucleotide-binding site (Nelson & Taiz, 1989). Despite these efforts, further information on the exact structure and the most essential amino acid residues

involved in the catalytic site of tonoplast ATPase, especially of higher plants, is still lacking.

Chemical modifiers have been widely employed as probes to identify essential amino acid residues in the active site, and to aid elucidation of the structure and properties of the enzyme active site. In this paper, we demonstrate that fluorescein 5'-isothiocyanate (FITC), a fluorescent probe specific for the modification of lysine residues, binds to the A-subunit and inhibits ATP hydrolysis and H⁺ translocation. Kinetic and labelling studies reveal that incorporation of approx. 1 mol of FITC/mol of ATPase is sufficient to inhibit the enzyme activity of ATPase. The FITC-modified lysine residue probably resides in a relatively hydrophobic domain of the active site.

MATERIALS AND METHODS

Materials

Seeds of *Vigna radiata* L. (mung bean) were allowed to imbibe tap water overnight and then germinated at room temperature in the dark. The hypocotyls of 6-day-old etiolated seedlings were excised and used as starting material.

FITC was obtained from Molecular Probes (Eugene, OR, U.S.A.) and dissolved in 50 mM-Tricine/NaOH (pH 7.5) as stock solution. All other chemicals were purchased from Sigma and E. Merck and used without further purification.

Preparation of tonoplast vesicles

After a rinse with deionized water, the excised hypocotyls were sliced into buffer I [75 mM-Tris/HCl (pH 7.6), 0.25 M-sorbitol, 5 mM-EGTA, 2 mM-dithiothreitol (DTT), 2 mM-phenylmethanesulphonyl fluoride (PMSF) and 0.25 mg of BSA/ml]. All the following procedures were carried out at 4 °C. The chilled hypocotyl slices were homogenized with a Polytron PT-35 at the medium speed setting for 2 min. The homogenate was filtered through four-layer cheesecloth and subjected to differential

Abbreviations used: DCCD, *NN'*-dicyclohexylcarbodi-imide; FITC, fluorescein 5'-isothiocyanate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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centrifugation at 480 *g* for 10 min and 6000 *g* for 15 min. The final pellets were redissolved in buffer II [5 mM-Tris/HCl (pH 7.3), 0.25 M-sorbitol, 1 mM-EGTA, 1 mM-DTT and 0.2 mM-PMSF]. The resuspension was layered over a 4% dextran (78 kDa) cushion for 2 h of centrifugation at 90000 *g*. The tonoplast-enriched band at the 0–4% interface was collected for the next step or stored at –70 °C for further experiments.

Solubilization and purification of tonoplast ATPase

Tonoplast vesicles were pretreated with deoxycholate in a medium containing buffer II, 15% (w/v) glycerol, 0.1% (w/v) deoxycholate and 1 mg of membrane protein/ml. After 30 min incubation at 4 °C, the tonoplast preparation was centrifuged at 120000 *g* for 1 h. The pellets were resuspended in a solution containing buffer II and 15% (w/v) glycerol to a protein concentration of 2 mg/ml and then 40 mM-octyl glucopyranoside was added dropwise. After 25 min incubation at 4 °C, the solution was centrifuged at 130000 *g* for 1 h; 1 ml of the supernatant was layered on to an 8–28% (w/v) linear sucrose gradient containing buffer II, 15% (w/v) glycerol and 0.2 mg of phosphatidylcholine/ml. The gradient was centrifuged at 200000 *g* for 3.5 h. Fractions (1.0 ml) with the highest ATPase activity were pooled for further studies.

Assay of enzyme activity and protein

Enzyme activities were assayed in a reaction medium containing 25 mM-Tris/HCl (pH 7.0), 3 mM-ATP, 3.0 mM-MgSO₄, 50 mM-KCl, 0.2 mg of phosphatidylcholine/ml, 1.0 mM-sodium vanadate, 0.5 mM-ammonium molybdate, 1.0 mM-sodium azide and approx. 30 µg of purified enzyme or membrane protein/ml. After a 30 min incubation at 33 °C, the reaction was terminated by adding a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) SDS and 0.02% (w/v) 1-amino-2-naphthol-4-sulphonic acid. The velocity of ATP hydrolysis was constant over the 30 min incubation period (results not shown). The released P_i was determined spectrophotometrically (Fiske & Subbarow, 1925; Wang *et al.*, 1989).

H⁺ translocation was measured as fluorescence quenching in a reaction mixture which contained 50 mM-Tris/HCl (pH 8.0), 250 mM-sorbitol, 10 mM-KCl, 3 mM-MgSO₄, 30 µg of vesicle protein/ml and 5 µM-Acridine Orange (Wang *et al.*, 1989). The excitation wavelength was 495 nm and the emission wavelength 530 nm. ATP (3 mM) was added to start the H⁺ translocation. After 5 min, 2 µg of gramicidin/ml was added to collapse the pH gradient.

Protein concentration was determined by a modified Lowry method (Larson *et al.*, 1986) with BSA as standard.

Chemical modification and labelling stoichiometry

Purified ATPase (250 µg) was incubated with 4.0 mM-FITC in 0.96 ml of buffer II for 10 min at 4 °C. Unchanged FITC was removed by passage through Sephadex G-25 by using spin filtration (Penefsky, 1977). Spin filtration of control ATPase did not cause any significant loss of enzyme activity under our conditions (results not shown). The modified enzyme was then used for enzyme assay or determination of stoichiometry.

The amount of FITC incorporated was determined by measuring absorbance at 495 nm with an absorption coefficient $\epsilon_{495} = 8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Pick & Karlish, 1980). FITC-ATPase was dissolved in a sample buffer containing 0.5 M-Tris/HCl (pH 6.8), 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 0.05% (w/v) Bromophenol Blue and 10% (w/v) SDS at 95 °C for 6 min. The mixture was then subjected to SDS/PAGE on a 12% (w/v) acrylamide slab gel (Laemmli, 1970). After electrophoresis, a strip of the gel was stained routinely and then scanned directly at 550 nm in an LKB Ultrascan XL densitometer (LKB,

Bromma, Sweden). The other unstained gel strips were cut into slices and separately homogenized. Polypeptides therein were then eluted into 1 ml of deionized water at room temperature overnight. The eluate from each gel slice was collected as the supernatant obtained by table-top centrifugation. The relative fluorescence intensity was measured by using appropriate excitation and emission wavelengths.

Kinetic analysis

$t_{1/2}$ values (time required for 50% inhibition of activity) at various concentrations of FITC were obtained from semi-logarithmic plots of percentage residual activity versus time as described by Levy *et al.* (1963). $T_{1/2}$ ($t_{1/2}$ at excess concentration of modifier) was calculated from the plot of $t_{1/2}$ versus the reciprocal of FITC concentration as described by Meloche (1967). Modification of ATPase by FITC follows the reaction in eqn. (1) (Borchardt & Thakker, 1975):

where E·FITC is the reversible complex, E-FITC the FITC-modified enzyme, k_{+2} the first-order rate constant. The reaction order (n) with respect to FITC was thus determined from double-logarithmic plots of the apparent rate constant, K_{obs} , versus concentration of modifier as follows (Meloche, 1967; Borchardt & Thakker, 1975):

$$\log K_{\text{obs}} = \log k_{+2} + n \log [\text{FITC}] \quad (2)$$

The steady-state constant of inactivation K_i , k_{-1}/k_{+1} , was estimated from the slope of the plot according to eqn. (3) (Carlson, 1984; Borchardt & Thakker, 1975):

$$\frac{1}{K_{\text{obs}}} = \frac{K_i}{k_{+2}} \times \frac{1}{[\text{FITC}]} + \frac{1}{k_{+2}} \quad (3)$$

Spectroscopic measurements

Absorption spectra of free FITC, ATPase and FITC-ATPase were determined in a medium containing 5 mM-Tricine/NaOH (pH 7.5), 10 mM-KCl and 3 mM-MgSO₄. Fluorescence measurements were carried out at 33 °C at an excitation wavelength of 495 nm.

RESULTS

Inactivation of ATPase by FITC

It has been reported that FITC is an extremely potent inhibitor of many types of ATPase, such as Na⁺/K⁺, H⁺/K⁺, Ca²⁺- and bacterial ATPases (Pardo & Slayman, 1988; Markus *et al.*, 1989; Tai *et al.*, 1989; Xu, 1989). In the first step of the present study the effects of FITC on the activities of purified and membrane-bound tonoplast H⁺-ATPase were examined. After incubation of tonoplast H⁺-ATPase with 0–300 µM-FITC, enzyme activity was markedly inhibited in a concentration-dependent manner (Fig. 1). The half-maximal inhibition (I_{50} values) occurred at 0.09 and 0.16 mM for soluble and membrane-bound ATPase respectively. At higher concentrations, the enzyme activities of both membrane-bound and soluble ATPase were completely inactivated. The sensitivity of soluble ATPase to FITC is greater than that of the membrane-bound enzyme indicating that the membrane might influence the accessibility of the active site to the inhibitor. We further demonstrated by a fluorescence-quenching technique that FITC decreased the rate and extent of H⁺ translocation across the vesicle membrane (Fig. 2). The concentration-dependence of inhibition of H⁺ translocation by

Fig. 1. Concentration-dependent curves of inactivation of ATPase by FITC

Membrane protein (2.0 mg/ml) and purified ATPase (0.5 mg/ml) were incubated for 10 min at 4 °C with various concentrations of FITC, as indicated, in a total volume of 20 μ l. After incubation, the reaction mixture was diluted to 1 ml with enzyme assay medium containing 25 mM-Tris/HCl (pH 7.0), 3 mM-ATP, 3 mM-MgSO₄, 50 mM-KCl, 0.5 mM-sodium vanadate, 0.1 mM-ammonium molybdate and 0.5 mM-sodium azide. Enzyme activity was assayed as described in the Materials and methods section. The effect of residual FITC, after dilution, on ATPase activity was negligible as compared with that using spin filtration. Control specific activities were 37.6 and 3.8 μ mol of P_i released/h per mg of protein for the purified and membrane-bound ATPase respectively. The reaction medium for fluorescence quenching contained 50 mM-Tris/HCl (pH 7.0), 250 mM-sorbitol, 50 mM-KCl, 3 mM-MgSO₄, 5 μ M-Acridine Orange and 35 μ g of membrane protein/ml. ATP (3 mM) was added to start the H⁺ translocation. Initial rates of fluorescence quenching were determined from changes in relative fluorescence observed during the first minute. The excitation wavelength was 495 nm, and the emission wavelength was 530 nm. Gramicidin (2.5 μ g/ml) was added to stop the reaction. ○, Control rates for H⁺ translocation and enzyme activities of ATPases; ■, membrane-bound ATPase; ●, soluble ATPase; ▲, ATP-mediated H⁺ translocation.

Fig. 2. Reaction traces of ATP-mediated H⁺ translocation inactivated by FITC

Inactivation of tonoplast ATPase by various concentrations of FITC and reaction conditions for H⁺ translocation were as described in Fig. 1.

Fig. 3. Fluorescence spectra of FITC-ATPase

ATPase (10 μ g/ml) was incubated with various concentrations of FITC for 10 min at room temperature. After incubation, free FITC was removed by spin-filtration through Sephadex G-25. The fluorescence spectra were measured at an excitation wavelength of 495 nm. Fluorescence was enhanced with increasing FITC concentrations of 0, 10, 50, 100, 150 and 200 μ M respectively from the bottom curve up. Inset: dependence of FITC-ATPase fluorescence at 517 nm on FITC concentration.

FITC was parallel to that of enzyme activity (Fig. 1). A similar I_{50} value of 0.17 mM was obtained for inhibition of both the H⁺ translocation and ATP-hydrolytic activity of vesicles by FITC.

Spectral properties of FITC-ATPase

After incubation of ATPase with various concentrations of FITC for 10 min in the dark at room temperature and the removal of unchanged FITC by passage through Sephadex G-25 by spin filtration, the absorption spectrum of FITC-ATPase was investigated. A characteristic absorption peak over the wavelength 440–540 nm appeared (results not shown). As the concentration of FITC increased, the intensity of the absorption maximum at 493 nm increased. The characteristic absorption peak around 493 nm was used for the stoichiometric studies (see below).

On the other hand, fluorescence over the range 500–600 nm appeared when ATPase was labelled with FITC (Fig. 3). The fluorescence intensity of FITC-ATPase increased with the increase in the number of FITC molecules incorporated per ATPase molecule (Fig. 3, inset). Furthermore, an obvious blue shift of emission maximum (from 522 nm to 515 nm) of FITC on incubation with ATPase was also observed (results not shown). The emission maximum of denatured FITC-ATPase was the same as that for free FITC. The blue shift of fluorescence maximum after modification indicates that the labelled FITC moiety is probably surrounded by a relatively more hydrophobic domain.

Fig. 4. Inactivation of enzyme activity of purified ATPase by FITC

(a) Time-courses of inactivation of purified ATPase with different concentrations of FITC. Purified ATPase (200 $\mu\text{g}/\text{ml}$) was incubated for the time-periods indicated at 4 $^{\circ}\text{C}$ as described in Fig. 1 with various concentrations of FITC: $\diamond$, none; $\square$, 15 μM ; $\circ$, 30 μM ; $\triangle$, 50 μM ; $\bullet$, 80 μM ; $\blacktriangle$, 100 μM ; $\blacksquare$, 200 μM . The enzyme reaction was carried out under the conditions described in Fig. 1. Control specific activities were approx. 39.2 μmol of P_i released/h per mg of protein. (a, inset) Apparent rate constant, K_{obs} , as a function of the reciprocal of FITC concentration. K_{obs} was plotted against FITC concentration according to eqn. (1). (b) Estimation of steady-state constant of inactivation, K_i , and first-order rate constant, k_{+2} . The double-reciprocal plot of apparent rate constant, K_{obs} , versus FITC concentration was obtained according to eqn. (2).

Fig. 5. Protection afforded by Mg^{2+} -ATP against FITC inactivation

ATPase (10 $\mu\text{g}/\text{ml}$) was preincubated with 5 mM- Mg^{2+} -ATP at 4 $^{\circ}\text{C}$ for 10 min. After pretreatment, 100 μM -FITC was added for the time-periods indicated. Enzyme activity was then measured as described in the Materials and methods section. Δ , Control ATPase activity; $\blacktriangle$ and $\bullet$, relative enzyme activities after inactivation by FITC with and without Mg^{2+} -ATP protection respectively. Control ATPase activity was 39.2 μmol of P_i released/h per mg of protein.

Kinetic analysis

Inactivation of ATPase by FITC as a function of time appeared to be pseudo-first-order according to semilogarithmic plots of percentage residual activity versus time (Fig. 4a). $t_{1/2}$ was obtained from the plots at each concentration. A further plot of $t_{1/2}$ versus reciprocal of the modifier concentration gives the minimum $t_{1/2}$ at infinite concentration of inhibitor, $T_{1/2}$ (Meloche, 1967). The

minimum inactivation half-time $T_{1/2}$ of 110 s was obtained from extrapolation of the straight line to the ordinate (results not shown). The K_i value of 0.33 mM for FITC was also calculated from the slope of the plot according to eqn. (3) (Fig. 4b) (Meloche, 1967; Borchardt & Thakker, 1975). The reaction order (n) and first-order rate constant (k_{+2}) were determined from a double-logarithmic plot of apparent rate constant, K_{obs} , as a function of modifier concentration according to eqn. (2) (Fig. 4a, inset). Accordingly, a straight line was obtained yielding a first-order rate constant of 0.244 s^{-1} and reaction order (n) of 0.913. The reaction order of 0.913 indicates that the inactivation of H^+ -ATPase was primarily due to modification of at least one lysine residue at the active site of the enzyme by FITC. It might be possible that more than one lysine residue exists at the active site. However, this possibility seems unlikely since the semilogarithm of percentage residual activity shows a simple exponential function of incubation time. In addition, the stoichiometry of labelling of the H^+ -ATPase by FITC excludes these alternatives (see below).

The effects on V_{max} and K_m were also investigated of incubating ATPase with or without FITC for 10 min at 4 $^{\circ}\text{C}$. In the control, the K_m for ATPase was 0.68 mM and V_{max} was 50 μmol of P_i released/h per mg of protein. In the presence of FITC, V_{max} was unchanged, but the K_m increased sevenfold to 4.77 mM (results not shown). The mode of inhibition of ATPase by FITC is competitive as determined by its effect on K_m but not V_{max} . It is thus confirmed kinetically that the modified lysine residue is involved in the enzyme activity and is probably located at the active site.

Protection against FITC-induced inactivation

Protection against FITC-induced inactivation was studied to demonstrate further the specificity of the inhibitor for the active

Table 1. Protection effect of ligands on inactivation of ATPase activity by FITC

ATPase was preincubated with 5 mM-ligands for 5 min at 4 °C in the presence or absence of 5 mM-MgSO₄. The enzyme was then inactivated for 10 min by addition of 0.1 mM-FITC in the presence or absence of protector(s) under the conditions described in Fig. 1. Samples of ATPase were diluted 25-fold in enzyme assay medium. Enzyme activity was assayed as described in the Materials and methods section. The hydrolytic activity of ligands as substrates was expressed as percentage of ATPase. Control ATP-hydrolytic activity was approx. 40.7 μmol of P_i released/h per mg of protein. The percentage protection was calculated according to the equation:

$$\text{Protection (\%)} = \frac{[\text{SA}_{(\text{protected})} - \text{SA}_{(\text{unprotected})}]}{[\text{SA}_{(\text{control})} - \text{SA}_{(\text{unprotected})}]} \times 100$$

where SA is specific activity. Each value is the mean of three assays with standard deviation less than ± 8 %.

Ligand (5 mM)	Activity as substrate (%)	Protection against 0.1 mM-FITC (%)	
		5 mM-MgSO ₄	0 mM-MgSO ₄
ATP	100.0	93.1	39.6
GTP	16.6	41.1	17.2
CTP	25.6	36.7	9.5
UTP	6.7	28.9	8.1
ADP	18.2	59.6	7.6
AMP	< 1.0	50.4	6.9
PP _i	12.3	69.9	4.3
<i>p</i> -Nitrophenol phosphate	< 2.3	35.7	13.3
P _i	—	27.0	8.5

site. ATPase was preincubated for 10 min at 4 °C with protectors in the presence or absence of 5 mM-MgSO₄ and 5 mM-ATP. After treatment with 0.1 mM-FITC for 10 min at 4 °C followed by a 25-fold dilution, ATPase activity was assayed. In the absence of ATP and MgSO₄, the activity was progressively inhibited as the incubation time with FITC increased (Fig. 5). However, in the presence of 5 mM-ATP and 5 mM-MgSO₄, more than 85 % ATPase activity was retained. Hydrolysis of ATP was negligible under the incubation conditions used. Table 1 summarizes the protective effects of the physiological substrate ATP, and P_i, PP_i, *p*-nitrophenol phosphate and several nucleotides. Substantial protection of the enzyme was provided by the physiological substrate ATP in the presence of 5 mM-MgSO₄. Addition of Mg²⁺ was crucial for partial protection against FITC. In its absence, the degree of protection by ligands was decreased. Other nucleotide triphosphates, which were poor substrates for ATPase, exerted weak protection in the presence of Mg²⁺. Protection by ATP was compared with that by the nucleotides listed in Table 1 with and without MgSO₄. The order of protection of nucleotides against inhibitor was ATP > ADP > AMP > GTP > CTP > UTP. It is possible that the difference in the degree of protection achieved by the different nucleotides is due to the steric fit of the base moieties of the physiological substrate ATP to the active pocket.

Furthermore, a protective effect by phosphate and its derivatives pyrophosphate and *p*-nitrophenol phosphate was observed. Pyrophosphate provided substantial protection (69.9 %) in the presence of Mg²⁺. However, phosphate and *p*-nitrophenol phosphate provided relatively poor protection (27.0 and 35.7 % respectively). The results suggest that FITC might modify the residue at a site far away from the binding domain for the γ-phosphate of ATP, but close to that for the ribose and base moieties.

Fig. 6. SDS/PAGE of purified ATPase labelled with FITC

Purified ATPase (250 μg/ml) was incubated with 0.1 mM-FITC in the presence or absence of 5 mM-ATP for 10 min at 4 °C. The detailed conditions for reaction and SDS/PAGE were as described in the Materials and methods section. (a) SDS/PAGE (12 %, w/v, acrylamide) revealed the normal pattern of ATPase subunits consisting of two prominent bands with molecular masses of 66 kDa (A-subunit) and 57 kDa (B-subunit). The dashed line represents the direct scan of the stained gel with a densitometer at 550 nm. The bar line represents the relative fluorescence intensity of labelled FITC. The excitation wavelength was 495 nm, and emission wavelength was 530 nm. (b) Relative fluorescence intensity of gel strip for FITC-ATPase pre-treated with Mg²⁺-ATP.

Location and stoichiometry of labelling

In order to determine which subunit of tonoplast ATPase was labelled, the modified ATPase was subjected to SDS/PAGE. The SDS/PAGE pattern shows two prominent bands with molecular masses of 66 kDa (A-subunit) and 57 kDa (B-subunit) and faint bands at 41 and 16 kDa. A separate gel was cut into 2 mm slices. The gel slices were then separately homogenized and the polypeptides therein were eluted overnight into 1 ml of deionized water at room temperature. Fluorescence intensities at 530 nm were measured for each eluate (Fig. 6). The fluorescence pattern of the gel revealed that the A-subunit of ATPase was primarily labelled with FITC (Fig. 6). In order to determine the specificity of the binding of FITC to the ATP-binding site, the ATPase was incubated with FITC in the presence of high concentrations of Mg²⁺-ATP. The extent of labelling of the A-subunit was markedly decreased. We are thus convinced that the active site of ATPase, which must be labelled by FITC, is located at the A-subunit.

The stoichiometry of labelling of H⁺-ATPase by FITC

Fig. 7. Stoichiometry of labelling of ATPase by FITC

Purified ATPase was incubated for 10 min with various concentrations of FITC and the residual activities and extent of ATPase labelling were determined as described in the Materials and methods section. Control specific activity was $41.5 \mu\text{mol of P}_i \text{ released/h per mg of protein}$.

was determined (Fig. 7). ATPase activity was inhibited to different degrees by different concentrations of FITC. The number of FITC molecules incorporated was determined by measuring absorbance with an absorbance coefficient, $\epsilon_{495} = 80000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Pick & Karlsh, 1980). The extent of labelling is represented as nmol of FITC/mg of protein. The degree of labelling paralleled the extent of inactivation, with complete inhibition occurring when approx. 2.6 nmol of FITC was incorporated/mg of H^+ -ATPase. With the protection afforded by Mg^{2+} -ATP, the inhibition of enzyme activity and the number of FITC molecules incorporated were decreased accordingly (results not shown). The molecular mass of purified tonoplast ATPase from higher plants has been reported to be in the range 400–600 kDa (Parry *et al.*, 1989; Matsuura-Endo *et al.*, 1990). The stoichiometry of labelling may therefore be calculated as approx. 1.0–1.3 mol of FITC/mol of ATPase.

DISCUSSION

In the present study we have demonstrated that FITC is an effective fluorescent probe for the ATP-binding site of tonoplast ATPase. FITC caused progressive inactivation of ATPase activity and its associated H^+ translocation. Inhibition of ATPase activity by FITC was competitive according to double-reciprocal plots. The minimum inactivation half-time was 110 s. Further kinetic studies yielded a steady-state constant of inactivation (K_i) of 0.33 mM and first-order rate constant (k_{+2}) of 0.244 s^{-1} . Kinetic and stoichiometry analysis indicated that approx. 1 mol of FITC was incorporated/mol of H^+ -ATPase. Inhibition of enzyme activity could be substantially prevented by the physiological substrate ATP in the presence of Mg^{2+} . The protection studies suggest that the modified lysine residue is probably in a region away from the binding site for the γ -phosphate of ATP but close to that for the ribose and base moieties. The SDS/PAGE pattern shows that FITC labelled the A-subunit of ATPase. FITC binding could be eliminated by the presence of a high concentration of Mg^{2+} -ATP. The above results suggest unambiguously that the catalytic site of tonoplast ATPase is located at the A-subunit and contains an essential lysine residue.

It has been shown that NEM and NBD-Cl can label tyrosine and cysteine residues on the catalytic (A) subunit of tonoplast ATPase and inhibit its enzyme activity (Manolson *et al.*, 1985; Mandala & Taiz, 1986; Schraw & Post, 1989). Furthermore, an

essential arginine residue at the active site of red beet ATPase has been demonstrated by using the site-directed reagent phenylglyoxal (Bennett & Borcherts, 1990). Several lines of evidence have shown that the carboxylate modifier, DCCD, can inhibit tonoplast ATPase by binding only at the 16 kDa component which is postulated to function as a proton channel. No trace of [^{14}C]DCCD was found in either the 66 kDa (A) or the 57 kDa (B) subunit. Whether the catalytic site on the A-subunit contains an essential carboxylate group remains to be determined. In this study, we have further revealed that the nucleotide-binding domain contains an essential lysine residue. The possible involvement of other crucial amino acid residues requires further investigation.

The primary amino acid sequence of the A-subunit of carrot (Zimnak *et al.*, 1988) and of both the A- and B-subunits of *Neurospora crassa* (Bowman *et al.*, 1988a,b) vacuolar ATPases have been deduced from cDNA sequences. The nucleotide-binding site of the A-subunit of carrot tonoplast ATPase is believed to be located in the region between residues 231 and 260, which are homologous with residues 129–158 of the β -subunit of *E. coli* F_1 -ATPase (Nelson & Taiz, 1989). This stretch of A-subunit of carrot tonoplast ATPase contains only one lysine residue, Lys-258. Although the amino acid sequence of tonoplast ATPase from mung bean has not yet been determined, we believe that the nucleotide-binding sites are also homologous with those of tonoplast ATPases of higher plants. It is likely that the lysine residue modified in this study (mung bean) is equivalent to that (Lys-258) of carrot A-subunit. However, we cannot exclude the possibility that the essential lysine residue labelled in this study is located on another fragment in the vicinity of the nucleotide-binding pocket. Further examination by peptide mapping is required to determine the alternatives.

The fluorescence enhancement and blue shift of emission maxima at 523 nm for the FITC-ATPase complex suggest that the FITC-binding site has a relatively hydrophobic environment. Some workers have demonstrated, using FITC and the fluorescent substrate analogue, that the essential lysine residue involved in ATP binding in Na^+/K^+ -ATPase is also embedded in a relatively hydrophobic pocket (Abbott *et al.*, 1991; Moczydlowski & Fortes, 1981). Whether the location of the essential lysine for ATP binding in a hydrophobic domain is a universal feature of ATPases deserves further investigation. Nevertheless, this conclusion is in good agreement with the protection studies, which reveal that the binding site of FITC is close to that of the base and ribose moieties rather than the more polar γ -phosphate end. The position of the essential amino acid residues, the architecture and properties of the catalytic pocket and the structure-function relationship of ATPase activity remain to be established.

The enhancement of absorption at 495 nm with concomitant loss of enzyme activity yields a stoichiometry of approx. 1 mol of FITC incorporated per mol of tonoplast H^+ -ATPase rather than per mol of catalytic (A) subunit. The molecular mass of tonoplast ATPase of higher plants has been estimated as 400–600 kDa (Parry *et al.*, 1989; Matsuura-Endo *et al.*, 1990). The stoichiometry of tonoplast ATPase is generally believed to be A_3B_3 similar to $\alpha_3\beta_3$ of F-type ATPase (Nelson, 1989; Tiedge & Schafer, 1989). For F-type ATPase, there are at least two very prominent models for the elucidation of the mechanism of ATP hydrolysis/synthesis, i.e. the binding change model proposed by Boyer (Kayalar *et al.*, 1977) and the non-equivalent (asymmetric) subunit model suggested by Wang (1984, 1985). The binding change model of Boyer suggests that the processes of ATP hydrolysis and synthesis involve three catalytic sites acting sequentially. However, one of the interesting features of F-ATPase, which led to the proposal of the non-equivalent subunit

model (Wang, 1984, 1985), is that it undergoes asymmetric modification of the β - (catalytic) subunit. In this report, we have demonstrated that tonoplast ATPase also shows asymmetric incorporation of FITC by the ratio of 1 mol of FITC per mol of ATPase rather than per mol of subunit, behaviour that is similar to that of F-type ATPase. We now encounter the question of whether the asymmetry is induced by chemical modification or whether it is a reflection of the inherent structure of the enzyme. At present there is insufficient evidence to support any of these possibilities. Further studies are required to elucidate the mechanism of ATP hydrolysis of V-type ATPase and to compare the similarities and differences in structure and function between V- and F-type ATPases.

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