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ATP-Induced Dynamic Fluorescence Changes of a *N*-[*p*-(2-Benzimidazolyl)Phenyl]maleimide Probe at Cys^{241} in the α-Chain of Pig Stomach H^+,K^+-ATPase[1]

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H^+,K^+-ATPase preparations from pig stomach were modified with a sulfhydryl fluorescence reagent, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide (BIPM). The addition of ATP to the modified enzyme preparations in the presence of Mg^{2+} decreased the BIPM fluorescence but increased the Trp fluorescence. After exhaustion of ATP, the fluorescence intensities increased and decreased to the original levels, respectively. The results of stopped flow and rapid quenching experiments suggested that the decrease in BIPM fluorescence (36/s) was accompanied by binding of Mg^{2+} and ATP or phosphorylation (35-36/s) which was followed by slower increases in Trp fluorescence (24/s) and light scattering (20/s). Tosylphenylalanyl chloromethyl ketone-trypsin treatment of the modified preparations, which showed an about 1% decrease in BIPM fluorescence accompanying phosphorylation, gave one major fluorescent peptide peak on reverse-phase chromatography. Amino acid sequence analysis of the peptide revealed the following sequence, Ser-Pro-Glu-X-Thr-His-Glu-Ser-Pro-Leu-Glu-Thr-Arg. On comparison with the amino acid sequence deduced from cDNA from pig stomach [Maeda, M., Ishizaki, J., and Futai, M. (1988) *Biochem. Biophys. Res. Commun.* 157, 203-209], X was shown to correspond to Cys^{241} of the α-chain in H^+,K^+-ATPase. These data and others suggest that the decrease in BIPM fluorescence at Cys^{241} reflects some molecular event triggered by the binding of ATP with Mg^{2+} and/or phosphorylation, whereas the increases in the intrinsic Trp fluorescence and light scattering reflect one after phosphorylation.

Key words: conformation change, fluorescence change, H^+,K^+-ATPase, rapid quenching, stopped flow.

The transport of H^+ and K^+ coupled with the hydrolysis of ATP is performed by H^+,K^+-ATPase (*1-8*). The kinetics of partial reactions of H^+,K^+-ATPase (*2-5*) suggest that ATP hydrolysis occurs *via* E_1P and E_2P as proposed in Na^+,K^+-ATPase (*9*) and Ca^{2+}-ATPase (*10*). The free energy of the ATP molecule appears to be converted to a change in the enzyme conformation, which induces the transport of H^+ and K^+ against their electrochemical gradients accompanying phosphorylation and dephosphorylation. Quite recently sequential phosphorylation of Tyr^{10} and Tyr^7 (*11*), and Ser^{27} (*12*) in the α-chain of pig stomach H^+,K^+-ATPase by membrane-bound kinases, and dephosphorylation by some phosphatases were shown unequivocally. The reversible phosphorylation may induce changes in the electrostatic interactions in the N-terminal domain for some regulatory mechanism (*6-8*).

To understand the molecular mechanisms of energy transduction and regulation of H^+,K^+-ATPase, detailed knowledge of conformational changes would be very useful. Conformational transitions around nucleotide binding domains have been identified on the basis of ligand-induced fluorescence responses of fluorescein 5′-isothiocyanate probe (*13*) at Lys^{517} (*14*) and eosin (*15*). Distinct fragmentation patterns for H^+,K^+-ATPase have been obtained on limited trypsin digestion with either ATP or KCl (*16*).

However, there seem to be few experimental data concerning conformational changes induced by ATP except ones concerning the increase in Trp fluorescence caused by ATP accompanying phosphorylation (*17*). A reversible fluorescence change of a voltage sensitive fluorescent dye bound to a membrane containing H^+,K^+-ATPase was reported recently to be due to changes in cation binding induced by phosphorylation (*18*). In this paper, we present evidence that the BIPM probe was incorporated at Cys^{241} of the α-chain without any influence on the H^+,K^+-ATPase activity, and that ATP in the presence of Mg^{2+} induced dynamic decreases in BIPM fluorescence and phosphorylation, with nearly the same apparent rate constant, followed by increases in Trp fluorescence and light scattering.

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Abbreviations: BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; TPCK, tosylphenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Vesicles containing pig stomach H^+,K^+-ATPase (G_1 fraction) were prepared, lyophilized, and stored in the presence of 250 mM sucrose containing 5 mM EGTA-Tris at −80°C (*19*): the specific activities of H^+,K^+-ATPase preparations in the presence of 2 mM $MgCl_2$, 25 mM sucrose, 0.1 mM EGTA-Tris, 40 mM Tris-HCl (pH 7.4), 16 mM KCl, and 1 mM ATP-Tris were 200 to 300 μmol P_i/mg/h at 37°C. Protein concentrations were determined with a Bio-Rad protein assay kit using bovine plasma albumin as a standard, and inorganic phosphate (*20*) liberated from ATP was determined as described (*17*). To modify the enzyme with BIPM, the H^+,K^+-ATPase preparations (1 mg protein/ml) were incubated with various concentrations of BIPM with 2% dimethylsulfoxide in a mixture containing 50 mM imidazole-HCl (pH 7.4), 0.1 mM EDTA-Tris, and 40% (v/v) glycerol at 25°C for 15 min in the dark. The modifications were stopped by dilution with 10 volumes of a buffer solution containing 25 mM imidazole-HCl (pH 7.4), 1 mM EDTA-Tris, and 25 mM sucrose containing various amounts of β-mercaptoethanol to give a 10-fold excess as to the amount of BIPM initially added. The modified enzyme preparations were collected by ultracentrifugation (Beckman-70 Ti, 45,000 rpm) for 30 min. The precipitates were washed one more time with the buffer described above, and then finally suspended (~5 mg/ml) in 250 mM sucrose and 0.5 mM EGTA-Tris, pH 7.4, and stored at −80°C until use.

The amounts of the BIPM probe bound to the α-chain were estimated using a molar absorption coefficient of 28,000 at 313 nm (*21*) as reported, except that a Superose-12 column (Pharmacia) was used to isolate the α-chain: the molar absorption coefficient of the chain at 280 nm was assumed to be 105,500, which was calculated from the sum of the coefficients of aromatic amino acid residues in the α-chain (*22*).

Steady-state fluorescence measurements were performed with a Shimadzu RF-503 difference spectrofluorophotometer (*23*). The fluorescence of Trp and BIPM was excited at 295 and 308 nm, respectively, and emitted light was detected at 325 and 360 nm, respectively. To determine the extent of the fluorescence change using a difference spectrofluorometer, 100 μg protein of BIPM-modified enzymes was suspended in 7 ml of a solution containing 2 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.4, 25 mM sucrose, and 0.1 mM EGTA-Tris. Fluorescence measurements were performed with an initial sample volume of 3.2 ml. The differences between the fluorescence changes induced by the addition of 5 μl of 6.4 mM ATP-Tris without or with 5 μl of 1,024 mM KCl to the sample cell, and that of ADP-Tris without or with 5 μl of 1,024 mM choline chloride to the reference cell are presented as percentage (Fig. 1B, closed circles); each fluorescence intensity value of 100% was taken from the relative fluorescence intensity of a sample before the addition of ATP. Transient-state fluorescence and light scattering measurements (*24*) were performed with an Applied Photophysics DX 17MV sequential stopped-flow spectrofluorometer at 25°C (*25*). The sample was excited at 295, 308, and 308 nm, and the emitted light was detected at 325.5, 308, and 355.7 nm, for Trp fluorescence, light scattering and BIPM fluorescence, respectively, after passage through Nippon Shinku Kogaku interference filters. The transient reactions were repeated at least 6 times under the same reaction conditions and the data were accumulated with a workstation (Archimedes 40/1). The ratios of accumulated data in the presence of specified ligands to those obtained in the controls were examined. The experiments were performed with different enzyme preparations and the data shown are for the same enzyme preparation unless otherwise stated. The signals were fitted as single or double exponentials. Time constants were estimated by non-linear least square fit analysis with the software supplied (Applied Photophysics).

To follow the time course of phosphorylation, rapid quenching experiments using a Quench-flow module (QFM-5, Biologic) were performed at 25°C by mixing equal volumes of a solution containing 40 mM Tris-HCl (pH 7.4), 0.1 mM EGTA-Tris, 25 mM sucrose, and 2 mM $MgCl_2$. In addition, 1 volume contained 320 μg protein/ml of the control enzyme or BIPM-treated enzyme, and the other volume contained 10 μM [^{32}P]ATP. After mixing, the reaction was quenched by adding one volume of ice-cold 15% trichloroacetic acid containing 15 mM P_i with 3 mM ATP. The denatured enzyme was washed on a glass fiber filter and then the amount of phosphoenzyme was determined as described (*26*). To determine the steady-state level of phosphorylation, the phosphorylation reaction was started at 25°C by adding 10 μl of 1 mM [^{32}P]ATP to 90 μl of a reaction mixture containing 25 mM imidazole-HCl (pH 7.4), 25 mM sucrose, 0.1 mM EDTA-Tris, and 15 μg of protein with 2 mM $MgCl_2$. The reaction was terminated at 10 s as described above.

To isolate BIPM-bound α-chains, 1.25 mg protein/ml of each enzyme preparation modified with various concentrations of BIPM was incubated with 100 mM phosphate buffer, pH 6.8, containing 2% SDS at 37°C for 20 min. The samples were then applied to a column of Sephacryl S-400 (3×50 cm) equilibrated with 100 mM sodium phosphate buffer containing 0.1% SDS (pH 6.8). The fractions containing the α-chain (as monitored as the absorption at 280 nm and the BIPM fluorescence) were pooled, concentrated, and desalted by membrane filtration (Amicon YM-5). After *S*-pyridylethylation as described (*27*), samples were applied on the same column as described above. Four volumes of cold acetone was added to the purified α-chain fraction. The sample was kept overnight at −20°C, and then the precipitate was collected by centrifugation and then dried under vacuum.

BIPM labeled α-chains (1-3 mg protein) were incubated (0.5 mg/ml) with TPCK-trypsin (1:3 w/w with respect to α-chain) obtained from Sigma in 25 mM Tris-HCl, pH 8.0, at 25°C for 4 h in the dark, and the same amount of TPCK-trypsin was added again for extensive overnight treatment. Digestion was terminated by the addition of formic acid at a final concentration of 2.7%, and samples were centrifuged at 100,000 rpm for 10 min. The supernatants were applied to a C_{18} reversed phase column of ODS-120T (Toyo Soda) equipped with a UV detector, monitoring the absorption at 215 nm, and a fluorescence detector, monitoring the BIPM probe (excitation at 313 nm and emission at 360 nm). The relative area of each fluorescence peak was calculated using a Chromato-pack (Shimadzu). Samples from the column were pooled and concentrated under vacuum.

Sequence analysis was performed with an Applied

Biosystem 477A gas-phase sequencer. PTH derivatives were identified with an Applied Biosystem 120A analyzer.

BIPM was obtained from Wako Pure Chemicals. [γ-^{32}P]ATP was purchased from Amersham, and all other chemicals used were of the highest grade commercially available.

RESULTS

Modification of H^+,K^+-ATPase by BIPM—It has already been shown that the BIPM probe at Cys^{964} in Na^+,K^+-ATPase (*26*) senses various conformational states of the enzyme (*23-26, 28-34*). The amino acid sequence of H^+,K^+-ATPase is highly homologous to that of Na^+,K^+-ATPase (*22*). These data suggested that the BIPM probe might also be useful as a conformational probe for H^+,K^+-ATPase. Incubation of H^+,K^+-ATPase preparations with 100 μM BIPM caused rapid incorporation of the BIPM probe into the α-chain (Fig. 1A, inset). The level of incorporation in 15 min at 25°C increased to ~2 mol/mol α-chain with increasing BIPM concentrations (Fig. 1A). The incorporation had little influence on the ATPase activity until ~1 mol BIPM probe/mol α-chain (Fig. 1B, open circles). However, the ATPase activity decreased with the increase in the incorporation from 1 to ~2 mol BIPM probe, with less effect on the phosphorylation capacity from ATP (Fig. 1B, squares).

When 10 μM ATP, in the presence of 1 mM Mg^{2+}, was added to BIPM-modified enzyme preparations containing 0.6 to 2.2 mol BIPM/mol α-chain, the BIPM fluorescence decreased immediately. A typical example is shown in Fig. 2A. The extent of the decrease induced by the addition of ATP increased biphasically with the increase in the amount of BIPM probe/α-chain (Fig. 1B, closed circles).

The BIPM fluorescence decrease due to ATP gradually increased with time to the level observed in the presence ADP, as shown in Fig. 2A. Readdition of ATP immediately decreased the fluorescence to nearly the same low level as observed after the initial addition of ATP (not shown). The addition of 0.5 mM K^+ together with 10 μM ATP reduced the BIPM fluorescence to nearly the same level as that induced by the addition of ATP alone, but induced a more rapid increase in the fluorescence to the initial level due to the acceleration of ATP hydrolysis.

The addition of ATP in the presence of Mg^{2+} to non-BIPM treated enzyme preparations increased the Trp fluorescence (Fig. 2B), as revealed by stopped-flow experiments (*17*). The fluorescence intensity decreased to the initial level after the exhaustion of ATP. The Trp fluorescence of the BIPM-modified enzyme also showed an increase induced by ATP and a decrease after the exhaustion of ATP, although the extent was reduced to half (Fig. 3B).

These data suggested that the dynamic changes in the

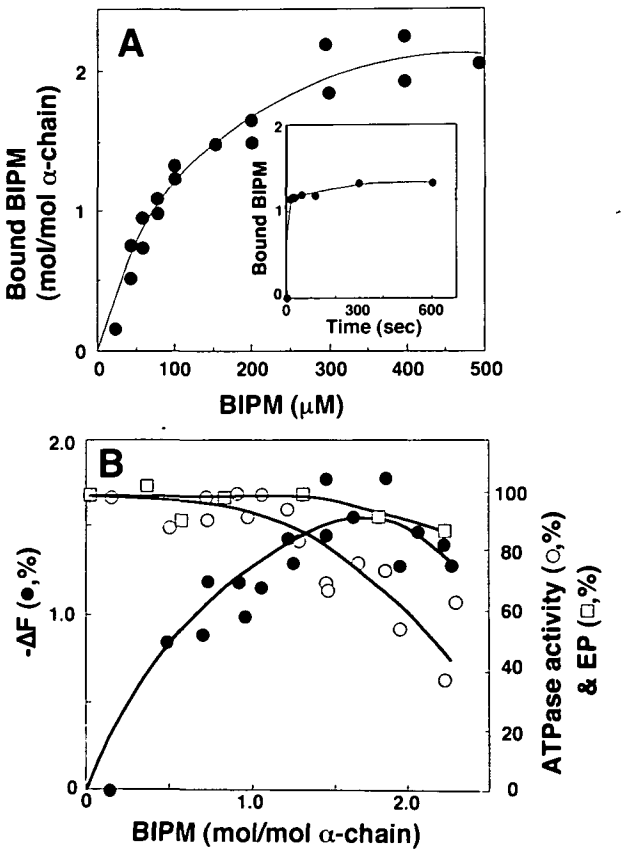

Fig. 1. **Effects of the BIPM concentration on the incorporation into the α-chain, the extent of the fluorescence change induced by ATP, and catalytic activities.** A: A H^+,K^+-ATPase preparation was incubated with the various concentrations of BIPM shown in the figure, and the amounts of bound BIPM (closed circles) were determined as described under "MATERIALS AND METHODS": the inset shows the time course of BIPM modification with 100 μM BIPM. B: The H^+,K^+-ATPase activity (open circles) and the amount of phosphoenzyme from ATP (squares), and the extent of the ATP-induced fluorescence change (closed circles) were determined as described. These data were obtained for several different enzyme preparations treated with various concentrations of BIPM.

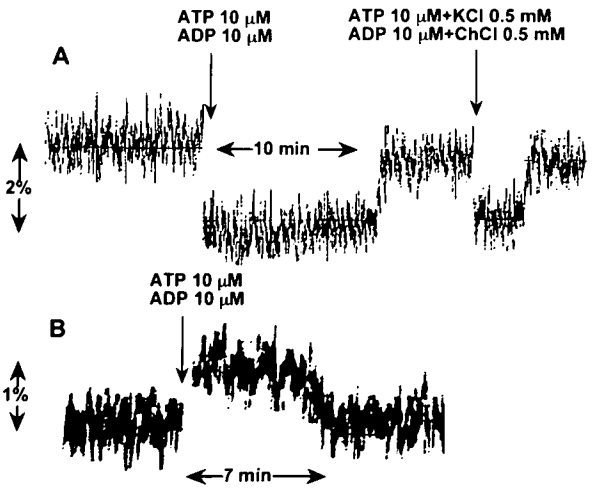

Fig. 2. **ATP-dependent reversible BIPM and Trp fluorescence changes.** The BIPM fluorescence of the BIPM-treated enzyme containing 1.5 mol BIPM probe/mol of α-chain (A), and the Trp fluorescence of the control enzyme (B) were measured as described above. The ligand additions were 5 μl of 6.4 mM ATP-Tris for the sample cell and 6.4 mM ADP-Tris for the reference cell. To investigate the effect of KCl on the ATP-induced BIPM fluorescence decrease, 5 μl of 320 mM KCl with 5 μl of 6.4 mM ATP-Tris, and 5 μl of 320 mM choline chloride (ChCl) with 5 μl of 6.4 mM ADP were added, respectively, to the sample and reference cells (A). The final concentration of each added ligand is shown in the figure. The time course runs from left to right.

fluorescence intensity of both BIPM and Trp reflected conformational events during H^+,K^+-dependent ATP hydrolysis, as already shown for Na^+,K^+-ATPase (*23-26, 28-34*). The incorporation of ~1 mol BIPM probe into the α-chain had little influence on either H^+,K^+-ATPase activity or the amount of phosphoenzyme from ATP, as shown in Fig. 1B.

Time Courses of Conformational Changes and Phosphorylation—To investigate the relationship between the fluorescence changes and phosphorylation further, stopped-flow and rapid quenching experiments were performed. The addition of ATP to the BIPM-enzyme in the presence of Mg^{2+} induced a single exponential decrease (Fig. 3A) in BIPM fluorescence (36/s). But it induced biphasic Trp fluorescence changes (Fig. 3B), *i.e.* a rapid decrease (33/s) followed by a slower increase (24/s). The Trp fluorescence of the enzyme preparations not treated with BIPM exhibited a single exponential increase with an apparent rate constant of around 25/s, as already reported (*17*). The data suggested that ATP in the presence of Mg^{2+} induced rapid decreases in both BIPM and Try fluorescence, which were followed by a slower increase in Trp fluorescence.

It has already been demonstrated that Na^+,K^+-ATPase preparations treated with BIPM show ATP-induced dynamic-light scattering changes accompanying the sequential appearance of species of ADP-sensitive phosphoenzymes that precedes K^+-sensitive phosphoenzyme formation (*24*). H^+,K^+-ATPase preparations treated with BIPM also showed an ATP-induced light-scattering increase (Fig. 3C), 20/s, which was clearly smaller than the rate constant of the BIPM fluorescence decrease.

To compare the rate of phosphorylation with the rate of conformational changes, the time course was followed. As shown in Fig. 3D, the phosphorylation of these enzyme preparations appeared to occur with nearly the same time course (35/s), irrespective of BIPM treatment. The rate constant was similar to those of the decreases in BIPM and Trp fluorescence, and clearly greater than that of the increase in the Trp fluorescence of the preparations treated with or without BIPM (*17*). The data clearly showed that the increase in Trp fluorescence occurred after phosphorylation.

Identification of a BIPM-Bound Residue—To identify the site of the bound BIPM probe that showed dynamic fluorescence changes accompanying phosphorylation and dephosphorylation, TPCK-trypsin was added to α-chains including, 0.35, 0.59, and 0.85 mol BIPM/mol α-chains obtained from H^+,K^+-ATPase preparations preincubated with 10, 50, and 100 μM BIPM, respectively. Up to 90% of the BIPM fluorescence of the α-chains became soluble on the digestion. The fluorescent peptides in the supernatant were separated by reverse-phase high performance liquid chromatography. Figure 4 shows the elution profiles of BIPM fluorescent peptides obtained from the sample containing 0.85 mol BIPM/mol α-chain. The data showed that the BIPM fluorescence was detected in several peptide peaks. The relative fluorescence intensity of each peak fraction obtained from each supernatant was calculated and is shown in Table I. The main fluorescent peptide peak was named No. 6, and appeared at a retention time of 57 min for

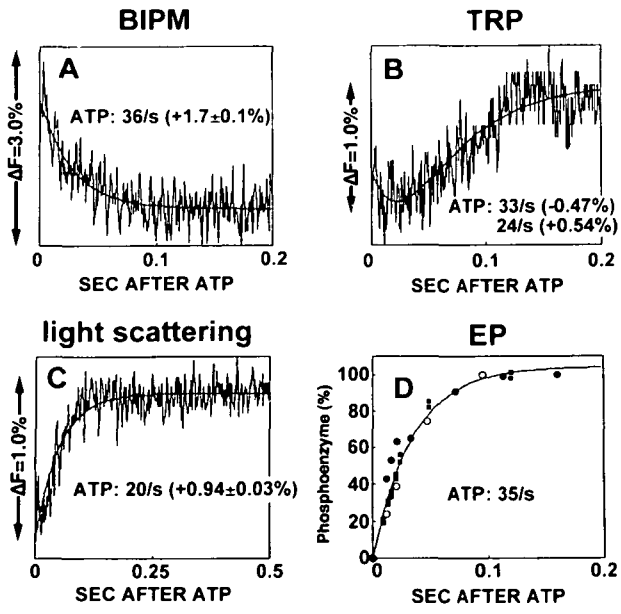

Fig. 3. **Stopped-flow measurements of ATP-induced changes in fluorescence and light scattering, and rapid quenching of [γ-^{32}P]ATP-induced phosphorylation.** The stopped-flow experiments were started by mixing equal volumes (25 μl) of the solution as described under "MATERIALS AND METHODS." In addition, one solution contained 20 μg/ml of enzyme protein and the other 20 μM ATP-Tris or choline chloride as a control. Reactions were started at 25°C as described in the text by the addition of ATP or choline chloride. The changes in BIPM fluorescence (A), Trp fluorescence (B), and light scattering (C) are expressed as the ratios of the data thus obtained. The phosphorylation reactions were started by the addition of [^{32}P]ATP and were stopped at various times as indicated. The amount of phosphoenzyme (D) was measured as described in the text. Different symbols (open circles and closed squares) indicate the results for different enzyme preparations and the BIPM-treated enzyme (closed circles) containing 1 mol BIPM probe/mol α-chain. The maximum amount of phosphoenzyme at 10 s was taken as 100%.

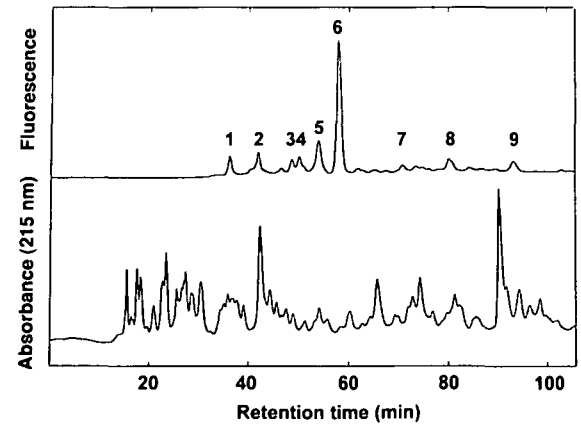

Fig. 4. **Elution profile of BIPM-labeled peptides on reverse-phase HPLC.** The supernatant of a tryptic digest of an α-chain preparation containing 0.85 mol BIPM/mol α-chain obtained from a sample preincubated with 100 μM BIPM was subjected to HPLC on a C_{18} reversed-phase (ODS-120T, Toyo-Soda) column (5 mm×250 mm). The column was developed with 0.1% trifluoroacetic acid containing a linear gradient of 0-6% acetonitrile for 5 min, and then one of 6-30% acetonitrile for 5-120 min, at the flow rate of 0.5 ml/min. The relative BIPM fluorescence intensity (excitation at 313 nm and emission at 360 nm), and the absorption at 215 nm *versus* retention time are shown (top and middle). The nine major BIPM fluorescent peaks are numbered from 1 to 9.

TABLE I. **Relative amount of the tryptic BIPM fluorescent peptide *versus* the amount of BIPM probe in the α-chain.** H^+,K^+-ATPase preparations were treated with 20, 50, and 100 μM BIPM as described. The resulting preparations contained 0.35, 0.59, and 0.85 mol BIPM/mol α-chain, respectively. Each supernatant of a tryptic digest of an α-chain preparation was applied to a HPLC column containing 0.1% trifluoroacetic acid as described in the text. The relative area of each peak (P_n) having BIPM fluorescence in Fig. 4 (top) was calculated with a Shimadzu Chromatopack using the following equation: $P_n \times 100\%/(P_1 + \cdots + P_2)$, where n is the number of each peak.

BIPM	Peak number								
	1	2	3	4	5	6	7	8	9
(μM)	(%)								
20	4.5	8.2	26.4	–	7.8	36.9	–	16.3	–
50	1.6	3.6	9.9	1.9	15.0	47.4	1.7	9.0	9.9
100	2.4	2.7	7.8	2.4	16.5	53.5	22.0	9.7	2.6

samples containing 0.35, 0.59, and 0.85 mol BIPM/mol α-chain. Peak No. 6 for the corresponding samples exhibited 37, 47, and 54% of the total fluorescence intensity, respectively (Table I). The fractions of other fluorescent peaks decreased or little changed with increasing amounts of the BIPM probe in the α-chain except for peak No. 5. Peak No. 5 obtained for the corresponding samples contained 8, 15, and 17% of the total fluorescence, respectively. The relative extent of the ATP-induced decrease in BIPM fluorescence seemed to be nearly constant, at -1%, or to increase slightly with the increase in the BIPM probe to around 1 mol/mol α-chain (Fig. 1B, closed circles).

To identify the binding site of the BIPM probe, a BIPM-treated enzyme preparation containing 0.8 mol/mol α-chain was digested as described above. The peak No. 6 material was isolated and subjected to amino acid sequence analysis. Its sequence was shown to be Ser-Pro-Glu-x-Thr-His-Glu-Ser-Pro-Leu-Glu-Thr-Arg (Table II). On comparison with the sequence deduced from cDNA of pig stomach (*22*), it was shown that the peptide began from Ser^{238} and ended at Arg^{250}, and X was shown to be Cys^{241} of the α-chain of H^+,K^+-ATPase. The peptide containing the BIPM probe at Cys^{241} was a typical tryptic peptide split between Arg^{237}-Ser^{238} and Arg^{250}-Asn^{251}.

TABLE II. **Amino acid sequence of the BIPM-labeled tryptic peptide.** The main BIPM fluorescent peptide, No. 6, obtained from a sample containing 0.80 mol BIPM probe/α-chain was applied to the same column with the same gradient system as in Table I except that trifluoroacetic acid was replaced with 5 mM sodium phosphate buffer (pH 6.6) containing 20 mM Na_2SO_4. A single BIPM fluorescent peptide peak fraction containing phosphate buffer was obtained. It was desalted and purified with the first gradient system containing 0.1% trifluoroacetic acid. The fluorescent peak material at 57 min was subjected to sequence analysis. The recovery of the isolated BIPM peptide from the BIPM-labeled α-chain was shown to be up to 20%.

Cycle	Amino acid		α-Chain amino acid residue
		(pmol)	
1	Ser	189	238
2	Pro	257	239
3	Glu	170	240
4	X	–	241
5	Thr	165	242
6	His	51	243
7	Glu	80	244
8	Ser	62	245
9	Pro	57	246
10	Leu	95	247
11	Glu	33	248
12	Thr	46	249
13	Arg	24	250
	End		

DISCUSSION

Several fluorescence peptide peaks were obtained even after extensive TPCK-trypsin treatment of the modified preparations (Fig. 4). The extent of the ATP-induced fluorescence change increased biphasically with the increase in the level of BIPM probe incorporation. The initial incorporation of $\sim$1 mol of BIPM neither influenced H^+,K^+-ATPase activity nor phosphorylation capacity from ATP, but further incorporation decreased the H^+,K^+-ATPase activity (Fig. 1B). These data suggested that the BIPM probe was incorporated at several sulfhydryl residues in the α-chain of H^+,K^+-ATPase. However, the ATP-induced fluorescence decrease in the samples containing 0.5 to $\sim$1 mol BIPM probe/mol α-chain (Fig. 1B) seemed to be nearly constant, $\sim$1%, or to increase slightly. The total BIPM fluorescence at Cys^{241} (F^{241}) was estimated by simple calculation.

F^{241} = [(relative fluorescence intensity, as shown as % in Table I) $\times$ (the amount of BIPM probe/α-chain)]. F^{241} increased from 27.7 ($=47\times0.59$) to 45.9 ($=54\times0.85$) with the increase in the amount of the BIPM probe from 0.59 to 0.85/α-chain. The total BIPM fluorescence at the unknown Cys residue of the peak No. 5 material was 8.9 and 14, respectively. These data suggested that Cys^{241} was rather specifically modified by the BIPM probe, and that the BIPM fluorescence at Cys^{241} of the α-chain showed dynamic changes during the H^+,K^+-ATPase reaction as shown above. If the BIPM probe at Cys^{241} was not the main probe that showed the dynamic fluorescence change, the extent of the fluorescence intensity change induced by ATP should decrease with increases in the amount of the BIPM probe in the α-chain. For the same reason, the BIPM probe in peak No. 5 seemed not to mainly contribute to the dynamic ATP-induced fluorescence change. More specific BIPM modification at Cys^{241} might be possible by pretreatment of H^+,K^+-ATPase preparations with *N*-ethylmaleimide to protect sulfhydryl groups from nonspecific BIPM modification. Such pretreatment was shown to be effective in the case of specific BIPM modification of Cys^{964} of the α-chain in Na^+,K^+-ATPase (*26*).

The extent of the ATP-induced fluorescence decrease increased to $\sim$1.5% with a further increase in the amount of the BIPM probe from 1 to $\sim$2 mol/α-chain accompanied by some decrease in the H^+,K^+-ATPase activity. Further experiments are required to identify the Cys residues inhibiting the H^+,K^+-ATPase activity and increasing the extent of the ATP-induced fluorescence change.

The peptide containing the BIPM probe at Cys^{241} was rather easily solubilized with TPCK trypsin (Fig. 4). The TPCK trypsin treatment could not solubilize peptides containing the BIPM probe at Cys^{964} (*26*). The BIPM probe at Cys^{964} is assumed to be near or in the transmembrane segment near the C-terminal (*36*), and its fluorescence change was shown to be sensitive to phospholipase-A treatment (*33*). The increases in both the fluorescence of the BIPM probe and Trp were observed accompanying accumulation of K^+-sensitive phosphoenzymes in Na^+,K^+-

ATPase (*28*). The opposite change of the BIPM fluorescence of H^+,K^+-ATPase ($\sim -1\%$) compared with that of Na^+,K^+-ATPase ($\sim +5\%$) seems to be due to the difference in the domain in which the BIPM probe was present (*22, 26*). The reason for the rather small decrease in BIPM fluorescence at Cys^{241}, $\sim 1\%$, accompanying phosphorylation of the H^+,K^+-ATPase may be due to its presence in the soluble domain and to nonspecific incorporation of BIPM probes into Cys residues other than Cys^{241} (Fig. 4). A similar amino acid sequence is present in the pig kidney Na^+,K^+-ATPase α-chain, Ser^{221} to Arg^{233} without Cys (*37*). Glu^{240}, Cys^{241}, His^{243}, and Ser^{245} in H^+,K^+-ATPase were replaced by Asp^{223}, Phe^{224}, Asn^{226}, and Asn^{228}, respectively, in Na^+,K^+- ATPase.

There seem to be at least two possible explanations for the biphasic Trp fluorescence change and the reduction of the final level of Trp fluorescence in the BIPM enzymes. One is that BIPM probes may accept fluorescence energy from the Trp residue accompanying phosphorylation in such a way as to induce both a rapid transient decrease in Trp fluorescence and a decrease in the extent of the Trp fluorescence. The apparent rate constants of ATP-induced fluorescence decreases of both the BIPM probe and Trp residue were similar, 33-36/s, which was also nearly the same as the rate constant of phosphorylation irrespective of BIPM treatment and clearly greater than that of the increase in Trp fluorescence, ~ 24/s, irrespective of BIPM treatment. Another is that the incorporation of the BIPM probe at Cys^{241} had a steric effect on the microenvironment of the Trp residue, as described above. The data seem to favor the former possibility, although the latter could not be excluded.

The present data suggested that the BIPM probe at Cys^{241} sensed phosphorylation at Asp^{386} in H^+,K^+-ATPase followed by an increase in Trp fluorescence (*17*). Cys^{241} seems to be present in the second cytoplasmic domain (*22*) between the 2nd and 3rd transmembrane segments, in which no Trp residues are present. The third cytosol domain contains 3 mol of Trp residues (*22*), and the site of phosphorylation at Asp^{386}, and ATP protectable sites for FITC binding at Lys^{518} (*15*) and PLP binding at Lys^{497} (*35*). These data may indicate that the second cytosolic domain containing Cys^{241} participates in molecular events such as phosphorylation and dephosphorylation, with the third cytosolic domain containing the phosphorylation site or close enough to permit fluorescence energy transfer from the Trp residue to the BIPM probe at Cys^{241}, which may cause a transient reduction of Trp fluorescence, as described above (Fig. 3C).

This is the first demonstration that ATP-induced conformational changes of H^+,K^+-ATPase in real time accompanying phosphorylation were followed by measuring changes in the BIPM fluorescence at Cys^{241} in the α-chain, the Trp fluorescence and the light scattering. Hydrogen ions should migrate from the inside to the outside of parietal cells accompanying the phosphorylation and dephosphorylation of H^+,K^+-ATPase. The slow increase in the Trp fluorescence after phosphorylation and the slower increase in light scattering may reflect such a process and/or oligomeric interaction required to complete the reaction cycle (*17, 38*). Further experiments are required to clarify these points.

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