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PHOSPHORYLATION OF HALF AND ALL SITES IN H^+,K^+-ATPASE RESULTS IN OPPOSITE CHANGES IN TRYPTOPHAN FLUORESCENCE

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The Trp fluorescence increased accompanying phosphorylation by ATP and Acetyl phosphate (AcP) in pig stomach H^+,K^+-ATPase, respectively, to maximum values of 0.86 and 0.37 %. The stoichiometry of the maximum amount of phosphoenzyme formed from ATP, that from AcP, that from inorganic phosphate (P_i), and the maximum amount of ATP binding to the enzyme was shown to be close to 1: 2: 2: 2. Phosphoenzymes formed were shown to be turning over. The addition of K^+ reduced the amount of phosphoenzyme from ATP to one-tenth but reduced those from AcP or P_i to only the half. The data show that the Trp fluorescence increased with phosphorylation of half of the sites but decreased with phosphorylation of all sites.

Transport of H^+ and K^+ coupled with the hydrolysis of ATP is performed by H^+,K^+-ATPase (1-4). The hydrolysis is assumed to be accompanied by conformational changes as distinguished by proteolytic digestion (5) or extrinsic fluorescence probes (6-10). Phosphorylation kinetics of H^+,K^+-ATPase by ATP (11) suggest that ATP hydrolysis occur via E_1P and E_2P as Na^+,K^+-ATPase and Ca^{2+}-ATPase (2,4,12,13). The enzyme also accepts P_i (14) or AcP (10) to form phosphoenzymes in amounts twice as large as from ATP (10,14). Kinetic studies have suggested the presence of two classes of substrate sites and simultaneous binding of both ATP and P_i to the H^+,K^+-ATPase (14-17). However the conformational differences between phosphoenzymes

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Abbreviations: AcP, acetyl phosphate; E1P, ADP sensitive phosphoenzyme; E2P, K-sensitive phosphoenzyme; FITC, Fluorescein 5'-isothiocyanate; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid.

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formed from ATP and those from AcP or Pi and the stoichiometries of the amount of phosphoenzyme and bound ATP have not been reported yet. Such information would be useful to elucidate the mechanism of energy transduction in ATPases, functionally dimeric enzymes (17).

Materials and Methods

Vesicles containing pig stomach H^+,K^+-ATPase were prepared, lyophilized and stored in the presence of 250 mM sucrose containing 5 mM EGTA-Tris at -80 °C (18): the specific activities of H^+,K^+-ATPase were 200 to 300 μmol/mg/hr at 37 °C. Concentrations of protein with bovine plasma albumin as a standard and P_i were estimated (19, 20). Phosphorylation reactions were started by adding [^{32}P]ATP, [^{32}P]AcP or [^{32}P]P_i at 25 °C and stopped after 10 sec by adding 1 ml of ice cold solution containing 5% trichloroaceticacid with 20 mM P_i and 10 mM nonradioactive substrate. The denatured enzyme was washed on a Whatman GF/F filter and the amount of phosphoenzyme was estimated (21).

The fluorescence measurements were performed using an Applied Photophysics DX 17MV sequential stopped-flow spectrofluorimeter (22) at 25 °C using the buffer solution for the phosphoenzyme formation described above. Tryptophan fluorescence was excited at 295 nm and the emitted light was detected after passage through a Nippon Shinku Kogaku interference filter (maximum transmittance at 325.3 nm).

Fluorescein 5'-isothiocyanate (FITC) treated enzymes were obtained by modification with 5 μM FITC at 25 °C (6).

To estimate the amount of bound ATP, the reaction mixture for the phosphorylation, in which 2 mM Mg^{2+} was replaced with 10 mM 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) -Tris (pH 7.4), were centrifuged at 100,000 rpm for 10 min at 2 °C using a 100.3 rotor in a Beckman Optima TI ultracentrifuge. The precipitates were suspended and counted. The specific binding was determined as the difference between the absence and presence of 1mM carrier ATP.

[^{32}P]ATP and [^{32}P] P_i were purchased from Amersham, and [^{32}P]AcP was synthesized (23).

Results and Discussion

It has been shown that dynamic changes in the Trp fluorescence occurred with phosphorylation and dephosphorylation in Na^+, K^+-ATPase (12) and Ca^{2+}-ATPase (13). However there is no report of Trp fluorescence changes accompanying phosphorylation in H^+,K^+-ATPase (4).

Addition of 50 μM ATP or 4 mM AcP to the enzyme induced single exponential increases in Trp fluorescence as shown in Fig.1A and B. Insets in Figs.1A and B show the concentration dependency of ATP and AcP on the extent and rate of increase. The addition of 4 mM P_i also increased the Trp fluorescence to give 0.2% (not shown). The data show that the increase by ATP was up to twice that by AcP or P_i.

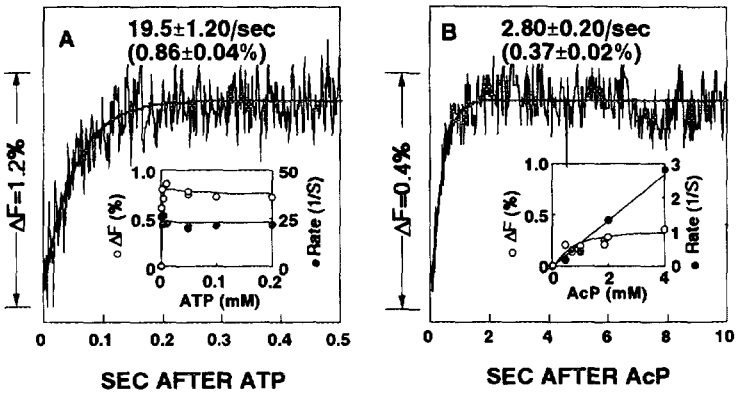

Fig. 1.Tryptophan fluorescence increases induced by ATP or AcP. Stopped-flow experiments on the fluorescence change at 25 °C were started by mixing equal volumes (25 ml) of a solution containing 40 mM Tris-HCl (pH 7.4), 25 mM sucrose, 0.5 mM EGTA-Tris and 2 mM $MgCl_2$, one volume contained 20 mg/ml enzyme protein and another volume contained 2 to 200 mM ATP-Tris (A inset) or 2 to 8 mM AcP-Tris (B inset) or the same concentrations of Tris-HCl as controls. The data were stored immediately after stopping the pistons and were accumulated at least 9 times.The ratios of the accumulated data in the presence of substrates to those obtained in the controls are shown. The starting positions of the curves were offset. The smooth lines show the data fitted to single exponential curves (22). The apparent rate constant and extent were19.5/s and 0.86% in the presence of 50 μM ATP (A) and 2.8/s and 0.37% with 4 mm AcP (B). Insets show the concentration dependence of ATP and AcP on the extent (open circles, ΔF) and the rate (solid circles) of the fluorescence increase.

To investigate the relationship between the relative fluorescence intensity and the amount of phosphoenzyme, the steady state levels of the phosphoenzyme were measured. The amounts of phosphoenzyme formed from 5 to 200 μM ATP, 4 mM acetyl phosphate, and 4 mM inorganic phosphate were, respectively, 2.4±0.1, 4.2±0.2 and 4.0±0.5 nmol/mg protein (Table 1, Fig.2). The data show that the amounts of

Table 1 Stoichiometry of the maximum amounts of phosphoenzyme and bound ATP

	Phosphoenzyme (nmol/mg)			Bound ATP (nmol/mg)	
	ATP	AcP	Pi	Mg^{2+}	CDTA
Native	2.4±0.1	4.2±0.3	4.0±0.5	<0.1	4.2±0.5
FITC	0.2±0.1	4.5±0.5	4.4±0.5	0.4±0.1	0.6±0.2

The protein (50 μg) treated without or with FITC was suspended in 180 μl of solution containing 2 mM Mg^{2+} and 40 mM Tris-HCl (pH 7.4). Phosphorylations were started by adding 20 μl of 500 μM [^{32}P]ATP, 40 mM [^{32}P]AcP, or 40 mM [^{32}P]P_i at 25 °C and stopped as described in the text. The ATP binding in the presence of 100 μM [^{32}P]ATP with 2 mM Mg^{2+} or 10 mM CDTA-Tris were also estimated as described in Fig.2. Data shown are the means and standard deviations of several experiments.

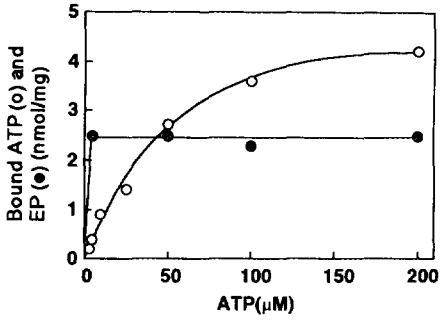

Fig.2.ATP binding to H^+,K^+-ATPase in the presence of CDTA. Before ultracentrifugation, 50 μg of enzyme protein was incubated with 40 mM Tris-HCl (pH 7.4) and 10 mM CDTA-Tris containing various concentrations of [^{32}P]ATP shown in the figure for 1min on ice. The amounts of phosphoenzyme in the presence of 5, 50, 100 and 200 μM ATP with 2 mM Mg^{2+} are also shown (solid circles).

phosphoenzyme formed from ATP were around half those formed from AcP or P_i (10,14).

Phosphoenzymes formed from [^{32}P]ATP or [^{32}P]AcP were chased by dilution with 20 volumes of solution containing the same concentration of ligands as the phosphorylation reaction except that the enzyme was absent and the substrates were replaced with non-radioactive ones. The data show that the formed phosphoenzyme were turning over.

To investigate the sensitivity of these phosphoenzyme, 50 mM KCl was added. The addition reduced the amounts of phosphoenzyme from ATP to around 1/10 but those from AcP and P_i to only half, which is nearly equal to the amount of phosphoenzyme from ATP. The remaining phosphoenzyme would be K^+-complexed phosphoenzyme (KE_2P) as known in Na^+,K^+-ATPase (24). The addition of ADP to phosphoenzyme formed from AcP influenced the rate of breakdown little, as reported for the phosphoenzyme from ATP (25).

The data and others (10, 14) suggest that both active site can be phosphorylated by AcP or P_i and either site by ATP. To investigate whether ATP binds to both sites, enzyme solutions containing various concentrations of [^{32}P]ATP with 10 mM CDTA-Tris were immediately subjected to the ultracentrifugation and the amounts of bound ATP were measured (Fig.2). They increased ($K_{0.5}$ = 50 μM) to give around 4.2 nmol/mg protein, nearly equal to the maximum phosphorylation capacities with AcP or P_i and almost twice that from ATP (Table 1).

The phosphorylation of half of the sites by ATP in the presence of Mg^{2+} suggests that phosphorylation of either subunit induced conformational change as reflected in the increase in Trp fluorescence. The change, possibly, in the non-phosphorylating subunit, prohibits ATP binding or transphosphorylation from bound ATP to Asp-385 (4). To investigate the effect of AcP on the half of the sites phosphorylation by ATP, the enzymes were phosphorylated in the presence of 125 μM ATP with or without 4 mM AcP in which either substrate was labeled with ^{32}P. The amounts of phosphoenzyme from [^{32}P]ATP, [^{32}P]ATP + AcP, ATP + [^{32}P]AcP, and [^{32}P]AcP were, respectively, 2.2, 1.2, 2.3 and 3.7 nmol/mg protein. The data show that the presence of both substrates gave 95% of the maximum amount of phosphoenzyme from AcP, which indicates that the half of the sites phosphorylated by ATP (1.2 nmol/mg protein) did not inhibit transphosphorylation from AcP to Asp-385 in the other subunit: if phosphoenzyme from ATP prohibits transphosphorylation to form phosphoenzyme in the other subunit, the amount of phosphoenzyme in the presence of both substrates would decrease to 1.3 nmol/mg protein (=3.7-2x1.2).

To investigate whether non-phosphorylating sites accept ATP , the amounts of [^{32}P]ATP bound to the enzyme in the presence of 100 μM ATP with 10 mM CDTA or 2 mM Mg^{2+} were measured. They were 4.2 nmol/mg protein in the presence of CDTA (Table 1) and 1.7 nmol/mg protein with Mg^{2+}. To determine whether ^{32}P was incorporated to the enzyme as aspartyl phosphate, 5% of trichloroacetic acid solution containing 2 mM ATP was added to the precipitates obtained in the presence of Mg^{2+}. The samples were centrifuged again and the amount of ^{32}P bound to the enzyme were measured to be around 1.6 nmol/mg protein. The data show that the amount of bound ATP was rather small, if present, only 0.1 nmol/mg protein or around 6 % of the amount of phosphoenzyme recovered by centrifugation irrespective of the addition of trichloroacetic acid.

The FITC treated Na^+,K^+- and Ca^{2+}- ATPases accept AcP and P_i to form phosphoenzymes but not ATP (12, 13, 22, 26) . Steady state measurements showed that the amounts of phosphoenzyme from AcP or P_i in FITC enzyme were nearly the same as those of the control enzyme (Table 1). But formation of phosphoenzyme from ATP and bound ATP in the presence of CDTA reduced to around 1/10. The data indicate that the FITC treatment strongly inhibit ATP binding as other p-type ATPase.

The maximum fluorescence increase by ATP was almost twice that by AcP (Figs. 1A and B). However the maximum amount of phosphoenzyme by ATP was half of that by AcP. The paradoxical opposite increase in the Trp fluorescence accompanying phosphorylation of half of the sites seems to be strong direct conformational evidence for the dimeric nature of the enzyme. It suggests that phosphorylation of either subunit induces conformational change in the non-phosphorylating subunit to prevent transphosphorylation from ATP to Asp-385 or that prevents binding of the bulky ATP molecules but not of AcP or P_i to form phosphoenzyme as detected. Both the rate and extent of the fluorescence increase with 1 to 200 μM ATP seemed similar (Fig. 1A inset). The amount of ATP detected in the presence of Mg^{2+} was negligible compared with that of phosphoenzyme. The data favor the possibility that phosphorylation of either subunit prevents binding of ATP to the non-phosphorylating subunit.

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