Microenvironment of Two Different Extrinsic Fluorescence Probes in Na⁺,K⁺-ATPase Changes Out of Phase during Sequential Appearance of Reaction Intermediates*

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Na⁺,K⁺-ATPase from pig kidney was sequentially modified with N-[p-(2-benzimidazolyl)phenyl]maleimide (BIPM) at Cys-964 and fluorescein isothiocyanate (FITC) at Lys-501. The resulting preparation showed little Na⁺,K⁺-ATPase activity with retention of nearly 90% of phosphorylation capacity from acetyl phosphate. The addition of acetyl phosphate to the preparation induced phosphoenzyme formation with a sequential decrease in the fluorescence intensities in the presence of 2 M NaCl and 4 mM MgCl₂; the BIPM fluorescence decreased with a simultaneous increase in the amount of phosphoenzyme; there was a significant delay in a decrease in the FITC fluorescence. The extent of the decrease in the BIPM fluorescence and the increase in the amount of phosphoenzyme both showed monophasic kinetics with a similar dependence on the concentration of acetyl phosphate (Kₐₑ = 4 mM), while that of FITC fluorescence showed a biphasic decrease (Kₐₑ > 10 mM). The phosphoenzyme formed was insensitive to ADP but sensitive to acetate (Kₐₑ = 2 M). These data and those of others (Taniguchi, K., Suzuki, K., Kai, D., Matsuoka, I., Tomita, K., and Iida, S. (1984) J. Biol. Chem. 259, 15228-15233) showed that the extent of the decrease in the BIPM fluorescence reflects an increase in the amount of a precursor of E₂P, and E₁P, respectively, of the FITC-treated enzyme. Therefore, it can be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Conformational changes during the Na⁺,K⁺-ATPase reaction have been suggested and shown from kinetic experiments of phosphorylation (1, 2), reactivity of sulfhydryl groups under various ligand conditions, sensitivity to proteolytic digestion, and extrinsic and intrinsic fluorescence probes (3-9). Direct conformational evidence for the Post-Albers mechanism has been shown clearly using BIPM-modified enzyme, in which Cys-964 of the α-chain was modified (10, 11). The BIPM fluorescence of NaE₁⁻¹ in the presence of Mg²⁺ decreased accompanying formation of E₁S* (Mg²⁺, Na⁺, and ATP-complexed enzyme). The formation of E₁P from E₁S* scarcely changed the fluorescence (10). The transition from E₁P to E₂P was accompanied by a maximal fluorescence increase (11). The formation of KE₂ from E₂P slightly reduced the fluorescence (12), and formation of E₁S* from KE₂ was accompanied by the maximum fluorescence decrease (13). Acceleration of Na⁺,K⁺-ATPase activity due to the acceleration of breakdown of KE₂ by high concentrations of ATP and ATP analogues (8, 9) has also been shown clearly (14). The possibility of the presence of functional low affinity ATP binding that accelerates the breakdown of E₁S* or E₂P was excluded (15). In experiments with fluorescein isothiocyanate-modified enzyme (5) at Lys-501 of the α-chain (16-18), a small increase and a large decrease in the fluorescence has been reported to be due, respectively, to the accompanying formation of E₁P (19) and various E₂ species such as KE₂, E₂P, and ouabain-E₂P (5, 6, 19). However, FITC-modified enzyme cannot accept ATP (5, 6, 19); thus, the correlation of the extent of fluorescence change with the amount of E₁P and E₂P has not been studied. Such correlation has only been clarified in BIPM-enzyme modified with BIPM at Cys-964 (10-12).

To compare the fluorescence changes in both BIPM and FITC probes, which are separated by 463 amino acid residues in the primary sequence (18), would be interesting for a better understanding of the conformational events that occur during the turnover cycle in this enzyme. Acetyl phosphate has been shown to phosphorylate the enzyme (20-22). Quite recently, it was clearly indicated that acetyl phosphate can replace ATP at the high affinity catalytic site sustaining active Na⁺-transport through the formation of the same phosphorylated intermediates obtained when the enzyme interacts with ATP (23). Na⁺-transport and the formation of these phosphoenzymes from acetyl phosphate using Na⁺,K⁺-ATPase preparations treated with FITC was also reported (19). We compare the fluorescence changes of these probes accompanying phosphorylation by acetyl phosphate using enzyme sequentially treated with BIPM and FITC or treated either with BIPM or with FITC alone.

This report shows that the fluorescence intensities of BIPM and FITC probes in BIPM-FITC-treated enzyme change out of the phase accompanying phosphorylation by acetyl phosphate in the presence of 2 M NaCl and 4 mM MgCl₂. The phosphoenzyme formed was shown to retain sensitivity to acetate but not to ADP.

MATERIALS AND METHODS

The Na⁺,K⁺-ATPase preparation from pig kidney purified by sodium deoxycholate (24) followed by NaI (25) has already been reported (11). The enzyme purified by sodium deoxycholate and sodium deoxycholate did not produce a preparation with satisfactory BIPM fluorescence response.
The specific activity of the preparations used was about 500–1000 μmol/mg of protein/h. The modification by BIPM at Cys-964 was performed as described with pretreatment with 64 μM N-ethylmaleimide (12). The resulting preparations retained almost full activity and were labeled with FITC as described for 15 min with 10 μM FITC at pH 9.2 and 25 °C (5) unless otherwise stated. The resulting double-labeled enzyme (BIPM-FITC-enzyme) preparations showed only 3–5% of Na,K-ATPase activity. The amount of FITC which became bound (26) to these enzyme preparations was around 60–70% of the amount of BIPM groups of the preparations.

[14C]Acetyl phosphate was synthesized from [14C]Pi, K2HPO4, and acetyl chloride as described (27) and was converted to the Tris form by passage through a column of Dowex 50W-X8 (Tris form).

The fluorescence measurements were performed by using a Shimadzu RF-503 difference spectrofluorophotometer and a Unisoku FSS-360 rapid mixing reaction apparatus at 25 °C as described (10, 11).

The phosphorylation reaction was started at 25 °C by adding 10 μl of various concentrations of [32P]labeled acetyl phosphate or [32P]ATP containing 40 nmol of MgCl2, 20 μmol of NaCl, 250 nmol of imidazole HCl (pH 7.4) to 10 μl of a reaction mixture containing the same solution as described above with 50–100 μg of protein of Na+,K+-ATPase, 1.6 μmol of succrose, and 5 nmol of EDTA-Tris (pH 7.4) except that the radioactive acetyl phosphate-Tris (or ATP-Tris) was omitted. After various time intervals, the reaction was terminated by adding 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM trichloroacetic acid. The acetyl phosphate-Tris or chaser by 1 ml of 100 μM EDTA-Tris (pH 7.4) containing 2 mM NaCl or sodium acetate (pH 7.4) with or without 1 mM ATP-Tris at 25 °C. The reaction was terminated by adding 1.25 ml of ice-cold 50% trichloroacetic acid containing 1 mM acetyl phosphate or ATP at various times after the chase. The denatured enzyme was washed (28), and the amount of phosphoenzyme was estimated as described (12).

FITC was obtained from Kodak, and acetyl phosphate and [32P]ATP were purchased from Sigma and Amersham Corp., respectively. BIPM was obtained from Wako Pure Chemicals, Ltd., Osaka. All other chemicals were of reagent grade.

RESULTS

Acetyl Phosphate-dependent Fluorescence Changes in Both BIPM and FITC Probes—Addition of ATP to the enzyme treated with BIPM and FITC (BIPM-FITC-enzyme) induced little fluorescence intensity change of either the BIPM or the FITC probe in the presence of NaCl and MgCl2 (Fig. 1, A and C). However, addition of acetyl phosphate to the BIPM-FITC-enzyme increased a rapid decrease in the fluorescence intensity of both probes (Fig. 1, B and D). Further addition of ouabain induced responses quite different from the fluorescence changes induced by acetyl phosphate: a slow increase and a slow and large decrease in the fluorescence intensity of BIPM and FITC probes, respectively.

Dependence of the Fluorescence Changes and Phosphorylation on the Concentration of Acetyl Phosphate—The BIPM fluorescence of the BIPM-FITC-enzyme showed a monophasic decrease with increasing concentrations of acetyl phosphate (K0.5 = 4 mM) to saturate around the ~3% level (Fig. 2B, open circles); a similar monophasic decrease of the BIPM fluorescence was observed with the BIPM-enzyme with increasing acetyl phosphate concentration (Fig. 2A, open circles) or by ATP with much higher affinity (11). The amount of phosphoenzyme of BIPM-FITC-enzyme also showed a monophasic increase (Fig. 2B, closed triangles) with similar affinity (K0.5 = 4 mM) for acetyl phosphate to that of the decrease in the BIPM fluorescence. The amount of phosphoenzyme of the BIPM-FITC-enzyme formed during 30 s in the presence of 40 mM [32P]acetyl phosphate was around 90 or 80% of that of non-FITC-treated BIPM enzyme formed under a similar condition using, respectively, 40 μM [32P]acetyl phosphate or 27 μM [32P]ATP (data not shown). The data suggest that the decrease in the BIPM fluorescence was correlated mainly with the formation of the phosphoenzyme. Addition of ouabain increased the BIPM fluorescence to 3% above the base level (Fig. 2A, closed circles).

Acetyl phosphate-dependent fluorescence intensity changes of BIPM and FITC probes in the presence of NaCl. Eighty μg of protein of the BIPM-FITC-treated enzyme were suspended in 7 ml of a solution containing 2 M NaCl, 4 mM MgCl2, 25 mM imidazole HCl, 25 mM sucrose, 0.1 mM EDTA-Tris, pH 7.4, at 25 °C. The fluorescence was excited at 315 or 494 nm, and emitted light was detected at 363 (BIPM) or 520 nm (FITC), respectively. The fluorescence measurement was performed with an initial sample volume of 3.2 ml. The solutions of ligand were 1 μM of 86.4 μM ATP (ATP) or 43 μl of 750 μM acetyl phosphate (AcP) and 100 μl of 15 mM ouabain (Ouab) to the sample cell and 45 μl of 750 mM Tris-HCl and 100 μl of H2O to the reference cell. The upward direction indicates an increase in fluorescence. The time course runs from left to right. The intensity of the initial solution at 365 and 520 nm was taken as a 100% value for the BIPM (A) and FITC (C, D) fluorescence, respectively.

Fig. 1. Acetyl phosphate-dependent fluorescence intensity changes of BIPM and FITC probes in the presence of NaCl. Eighty μg of protein of the BIPM-FITC-treated enzyme were suspended in 7 ml of a solution containing 2 M NaCl, 4 mM MgCl2, 25 mM imidazole HCl, 25 mM sucrose, 0.1 mM EDTA-Tris, pH 7.4, at 25 °C. The fluorescence was excited at 315 or 494 nm, and emitted light was detected at 363 (BIPM) or 520 nm (FITC), respectively. The fluorescence measurement was performed with an initial sample volume of 3.2 ml. The solutions of ligand were 1 μM of 86.4 μM ATP (ATP) or 43 μl of 750 μM acetyl phosphate (AcP) and 100 μl of 15 mM ouabain (Ouab) to the sample cell and 45 μl of 750 mM Tris-HCl and 100 μl of H2O to the reference cell. The upward direction indicates an increase in fluorescence. The time course runs from left to right. The intensity of the initial solution at 365 and 520 nm was taken as a 100% value for the BIPM (A) and FITC (C, D) fluorescence, respectively.

Fig. 2. Dependence of the fluorescence changes and phosphorylation on the concentration of acetyl phosphate. Eighty μg of protein of the FITC-treated (FITC-ENZYME), the BIPM-treated (BIPM-ENZYME), or the BIPM-FITC-treated enzyme (BIPM-FITC-ENZYME) were suspended as described in Fig. 1, except that the volumes of acetyl phosphate solution varied from 4.2 to 206 μl. The amounts of phosphoenzyme in the presence of 2 M NaCl and 4 mM MgCl2 (closed triangles) at 30 s after the addition of [32P]acetyl phosphate were also measured. The amount of phosphoenzyme in the presence of 40 mM acetyl phosphate was 640 ± 50 pmol/mg protein, which value was taken as the 100% value. A, acetyl phosphate-induced fluorescence intensity changes of BIPM (○) and FITC (♦) probes of BIPM-treated (BIPM-ENZYME) and FITC-treated (FITC-ENZYME) enzyme, respectively. B, acetyl phosphate-induced accumulation of phosphoenzymes (A); the standard deviation of triplicate experiments is within the closed triangles; fluorescence intensity changes of the BIPM (○) and FITC (♦) probes in the BIPM-FITC-enzyme before (○, ♦) and 25 min after the additions of ouabain to form ouabain-EaP (■, ■) are shown. Each bar indicates the standard deviation of experiments repeated 3–7 times.
line, irrespective of the concentration of acetyl phosphate at or above the lowest concentration used (Fig. 2B, open squares). A similar decrease and increase in the BIPM fluorescence in the BIPM-enzyme produced by ATP have been shown to occur accompanying the formation of the precursor of EiP, namely EiS*, which has nearly the same relative fluorescence intensity as EiP (10) and ouabain-EiP, respectively (11). The FITC fluorescence showed a biphasic decrease (Fig. 2B, closed circles) with increasing concentrations of acetyl phosphate to reach a level around −7% (K93 > 10 mM). Nearly the same concentration dependence was observed with the enzyme treated with FITC only (Fig. 2A, closed circles). The addition of ouabain further reduced the fluorescence intensity to give a level around −35% (Fig. 2B, closed squares). A similar decrease also occurred in the fluorescence of the FITC probe produced by ouabain in the enzyme treated with FITC only (data not shown). These data show that the BIPM-FITC-enzyme accumulated some phosphoenzyme which showed a similar relative BIPM fluorescence intensity to that of EiP formed in the BIPM-enzyme (11) and showed a large decrease in FITC fluorescence, relative to that of the Na∗ form of the enzyme.

Time Course of Fluorescence Changes and Phosphorylation—Fig. 3, A and B, shows the effect of the additions of acetyl phosphate on the time course of fluorescence changes of both BIPM and FITC probes of the BIPM-FITC-enzyme in the presence of 2 mM NaCl and 4 mM MgCl2. A monophasic decrease in the BIPM fluorescence, which was accelerated by increasing the concentration of acetyl phosphate, was observed (Fig. 3A). A similar acceleration of a monophasic decrease in the BIPM probe fluorescence in BIPM enzyme was produced by ATP; this concentration dependence was previously attributed to a large dissociation constant for ATP of a precursor of EiS* (15). A simultaneous addition of acetyl phosphate with K* induced a biphasic response in the BIPM probe in the BIPM-FITC-enzyme (Fig. 3A). A similar biphasic change induced by ATP with K* was also observed in the BIPM enzyme and was attributed to the formation of EiS*, EiP, EiP, and KE2 in sequence (10).

The time courses of FITC fluorescence change showed clear differences from that of BIPM fluorescence change in the dependence of the rate of fluorescence change on the concentration of acetyl phosphate (Fig. 3, B and A); the sigmoidal decrease in the extent of the fluorescence was already shown in the steady state measurements (Fig. 2B, closed circles). The data suggest the presence of at least two different phosphoenzymes with the same relative BIPM fluorescence intensity but with different relative FITC fluorescence intensity, because the amount of phosphoenzyme was nearly saturated at or above 10 mM acetyl phosphate irrespective of the relative fluorescence intensity of FITC probe (Fig. 2B). The addition of K* with acetyl phosphate further decreased the fluorescence intensity, which suggests the accumulation of dephosphoenzyme, namely KE2.

It has already been shown that the addition of 27 μM ATP to BIPM enzyme in the presence of 2 mM NaCl with 0.43 mM MgCl2 induced the same time course of the formation of both EiP and a decrease in the BIPM fluorescence (10). When Mg2+ was replaced with Ca2+, a rapid decrease in the BIPM fluorescence preceded the formation of EiP, which shows clearly that the decrease in the BIPM fluorescence is due to the formation of a precursor, EiS* (10). To investigate the reason for the decrease in BIPM fluorescence induced by acetyl phosphate in the BIPM-FITC-enzyme, the time course of phosphoenzyme formation was compared with both BIPM and FITC fluorescence changes. The increase in the amount of phosphoenzyme (Fig. 3C, closed triangles) agreed well with the decrease in the BIPM fluorescence. The data show clearly the presence of phosphoenzymes with different relative FITC fluorescence intensities.

Sensitivity of the Phosphoenzyme to ADP and Acetate—It has already been shown that the phosphoenzyme accumulated in the presence of 2 mM NaCl is EiP, irrespective of modification of the enzyme with BIPM (11, 29, 30). FITC treatment caused a strong reduction in Na∗,K∗-ATPase activity because ATP binding activity was lost (5, 6, 19). However, the results of the present experiments clearly demonstrate an accumulation of phosphoenzyme from acetyl phosphate, of which the conformational state seems to be EiP, as judged from the BIPM fluorescence (10–12). To investigate this point further, the sensitivities of the phosphoenzyme to ADP and acetate were investigated, because EiP is known to react with ADP (2, 8, 9, 11, 29) and because acetyl phosphate can phospho-rylate the enzyme and acetate should be liberated during turnover. Table I shows that the phosphoenzyme formed from the BIPM-enzyme was split by ADP very easily as already shown (11) and 2 mM acetate reduced the phosphoenzyme level nearly to half. However, FITC treatment of the BIPM-enzyme caused almost complete loss of the sensitivity to ADP without changing the sensitivity to acetate.

The time course of the breakdown of phosphoenzyme by sodium acetate was also compared with that by NaCl (Fig. 4). In these experiments, phosphoenzyme with two different relative FITC fluorescence intensities was kinetically isolated; the enzyme was phosphorylated in the presence of 10 mM acetyl phosphate for 3 s or in the presence of 40 mM acetyl

![Fig. 3. Time course of acetyl phosphate-induced fluorescence changes in the BIPM-FITC-enzyme in the presence of 2 mM NaCl.](image-url)
TABLE I
Reactivity of ADP and acetate to the phosphoenzyme in BIPM-FITC-treated enzyme

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Fig. 4. Time course of phosphoenzyme breakdown by sodium acetate. Phosphoenzymes formed in the presence of 10 mM acetyl phosphate were chased with EDTA and 2 mM NaCl. The amounts of EP remaining were, respectively, taken as 100% of the amount of phosphoenzyme. The values 2 s after the chase are shown. The amounts of phosphoenzyme remaining were, respectively, taken as 100% of the amount of phosphoenzyme.

It is reasonable to suggest that the difference between the time course of the BIPM fluorescence change and that of FITC may be due to the distribution of unevenly modified FITC residues in the BIPM-modified enzyme. The doubly labeled enzyme preparations retained only 3-5% of Na+K+-ATPase activity and showed little fluorescence change induced by ATP. However, the addition of acetyl phosphate induced significant fluorescence changes, with nearly 90% of the phosphorylation level from acetyl phosphate as described.

These data also suggest that the conformational changes in Na+K+-ATPase initiated by the formation of E1P (10) are transmitted to different domains in a sequential rather than a simultaneous manner. To investigate the changes in the distance between these residues during turnover would be a prerequisite for understanding the conformational change quantitatively.

DISCUSSION

Iodinated derivatives of fluorescein specifically inhibit nucleotide binding to lactate dehydrogenase (31) and yeast hexokinase. FITC is thought to react somewhere in the nucleotide binding site of Na+K+-ATPase, because ATP-dependent functions of the enzyme with FITC bound at Lys-501 are abolished and ATP strongly prevents the binding which abolishes these functions (5, 6, 16-19, 26). The present data support the idea with a finding that the FITC labeling little affected the acetyl phosphate-induced conformational state of the enzyme which was detected by fluorescence changes of BIPM at Cys-964 (Fig. 2, A and B, Fig. 3A, and text). The phosphoenzyme formed by acetyl phosphate in FITC-BIPM-enzyme in the presence of 2 mM NaCl with 4 mM MgCl2 was shown to be conformationally E2P without ADP sensitivity but with an acetate sensitivity (Table I) similar to that of the E1P of the BIPM-enzyme (Fig. 2, A and B, Fig. 3A, and text).

These data and those of others (10) show that the microenvironment of BIPM probes at Cys-964 changes first, accompanying E2S* formation followed by E2P formation with little change in that of FITC probes at Lys-501. Then the microenvironment of FITC probes gradually changed. Addition of ouabain E1P produced ouabain E1P, in which both microenvironments further changed. The dependence on the concentration of acetyl phosphate of the fluorescence changes of both probes accompanying E1P formation was different; the K0.5 for acetyl phosphate for the BIPM fluorescence was 2-4 mM, irrespective of FITC modification of the BIPM-enzyme, and around 10 mM for the FITC fluorescence, irrespective of FITC modification of Na+K+-ATPase (Fig. 2, A and B). This suggests that the two residues are not present in the same domain of the three-dimensional structure in Na+K+-ATPase.

It was already reported that each microenvironment of the BIPM probe at Cys-964 and of tryptophan residues (11, 12) and of 2 mol of N-7-dimethylamino-4-coumarinyl maleimide groups (30), the binding sites of which are not identified in the primary sequence, also changes out of phase during turnover (11, 12, 31). The present data clearly show that both microenvironments of fluorescence probes at Cys-964 and Lys-501 change out of phase accompanying phosphorylation. These data also suggest that the conformational changes in Na+K+-ATPase initiated by the formation of E1P* (10) are transmitted to several domains in a sequential rather than a simultaneous manner. To investigate the changes in the distance between these residues during turnover would be a prerequisite for understanding the conformational change quantitatively.

It is reasonable to suggest that the difference between the time course of the BIPM fluorescence change and that of FITC may be due to the distribution of unevenly modified FITC residues in the BIPM-modified enzyme. The doubly labeled enzyme preparations retained only 3-5% of Na+K+-ATPase activity and showed little fluorescence change induced by ATP. However, the addition of acetyl phosphate induced significant fluorescence changes, with nearly 90% of the phosphorylation level from acetyl phosphate as described. These data indicate that nearly 95-97% of the enzyme preparation contained at least 1 mol of FITC probe/a-chain, which caused the inhibition of not only ATP binding (5, 6, 19, 26) but also ADP binding to E1P (Table I). It has been clearly shown that the time course of inhibition of Na+K+-ATPase activity at each concentration of FITC fitted to a first-order rate equation (16). The incorporation of FITC seemed to be very specific, because the trypsin-digested samples showed only one large peak of FITC fluorescence in a reverse-phase high performance liquid chromatography column (16, 17) and because a preparation labeled with FITC for 1.5 min also showed similar fluorescence changes (data not shown) to those of the preparations treated for 15 min as in this paper. The peptides reported to be sequenced by two laboratories are the same lysine residues as those at the FITC site (16, 17). However, it has also been reported that prolonged incubation with 10 µM FITC (>2 h) induced the incorporation of another FITC molecule, which caused the inhibition of both K+-phosphatase and phosphorylation activity (5). The modifica-
tion of the BIPM-enzyme with FITC (10 μM for 15 min) reduced Na⁺,K⁺-ATPase activity below 5% with retention of 90% of phosphorylation capacity, which suggests the presence of FITC groups other than that at Lys-501. Their quantity may be around 10% of that of the active site. From these results we concluded that the difference in the time course of fluorescence of BIPM and FITC probes and the sigmoidal decrease with respect to concentration of acetyl phosphate are due to the presence of E₁Ps with a different relative fluorescence intensity; both the amount of phosphoenzyme and the extent of fluorescence change of the BIPM probe were almost saturated at or above 10 mM acetyl phosphate irrespective of the relative fluorescence intensity of FITC probe as described above and because the reactivity of acetate to the phosphoenzyme was also the same irrespective of the relative fluorescence intensity of FITC probe (Fig. 4, closed circles and squares). Sequential appearance of E₁Ps has already been shown by the reversible changes in the light-scattering intensity (30). Recently the presence of another EP other than E₁P and E₂P has also been discussed (34–36). To study the conformational state of such EPs seems to be indispensable for the understanding of the mechanism.

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