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Evidence for a Relationship between Activity and the Tetraprotomeric Assembly of Solubilized Pig Gastric H/K-ATPase

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Activity-oligomeric assembly relationships using octaethylene glycol dodecyl ether ($C_{12}E_8$) solubilized pig gastric H/K-ATPase (unmodified H/K-ATPase) or H/K-ATPase modified with Fluorescein 5′-isothiocyanate (FITC-H/K-ATPase) were examined. The amount of oligomeric species in FITC-H/K-ATPase, which retained little H/K-ATPase activity was estimated by a single-molecule detection technique using total internal reflection fluorescence microscopy. Solubilization of the FITC-H/K-ATPase reduced the potassium-dependent *p*-nitrophenyl phosphatase (K-pNPPase) activity to around 5% of the level of the membrane-bound enzyme with the formation of 50% protomer and 40% diprotomer. The solubilization of unmodified H/K-ATPase also reduced both the K-pNPPase and H/K-ATPase activities to around 5%. However, solubilization with increasing concentrations of potassium acetate induced significant and similar increases in K-pNPPase activity ($K_{0.5}$ = 35 mM) with an increase in the amount of the tetraprotomer of FITC-H/K-ATPase, and the K-pNPPase ($K_{0.5}$ = 28 mM) and H/K-ATPase ($K_{0.5}$ = 40 mM) activities of the unmodified H/K-ATPase. The correlation coefficient between the proportion of tetraprotomer and the proportion of the K-pNPPase activity for the same FITC-H/K-ATPase preparation was estimated to be 0.93. Similar coefficients were also obtained between the proportion of tetraprotomer in the FITC-H/K-ATPase and the proportion of K-pNPPase and H/K-ATPase activities in the unmodified H/K-ATPase, with value of 0.85 and 0.86, respectively. Such positive correlations were not obtained between these activities and other oligomeric species. These data, the first direct comparison of oligomeric assembly and enzyme activity both stabilized by K^+ in $C_{12}E_8$-solubilized gastric H/K-ATPase, provide strong evidence that the catalytic unit of $C_{12}E_8$-solubilized gastric H/K-ATPase is a tetraprotomer.

Key words: H/K-ATPase, oligomeric enzyme, single-molecule observation, tetraprotomer, TIRFM.

Abbreviations: H/K-ATPase, gastric potassium–activated adenosine triphosphatase; EP, phosphoenyzme; EATP, enzyme-bound ATP; P_i, inorganic phosphate; TIRFM, total internal reflection fluorescence microscopy; $C_{12}E_8$, octaethylene glycol dodecylether; OG, *n*-octyl glucoside; pNPP, *p*-nitrophenyl phosphate; K-pNPPase, K^+-dependent *p*-nitrophenyl phosphatase.

Gastric proton transporting H/K-ATPase (*1–3*) is a member of the ion-motive and phosphorylating P_2-type family of ATPases that includes sarcoplasmic reticulum Ca-ATPase (SERCA1a) and Na/K-ATPase (*4–7*). The electroneutral exchange of extracytoplasmic K^+ for cytoplasmic H^+ is achieved by utilizing the free energy of ATP hydrolysis. H/K-ATPase is composed of a 100 kDa catalytic α-subunit and a 35 kDa sialoglycosylated β-subunit (αβ-protomer). The transport cycle of H/K-ATPase is accompanied by a conformational change induced by high affinity ATP binding, phosphorylation and dephosphorylation of the Asp-386 catalytic subunit, and subsequent low affinity ATP binding to repeat the cycle (*8*).

There are long standing debates concerning the structure of P-type ATPases, namely whether a protomer is the functional unit of the enzyme or an oligomeric structure is required (*8–23*). It is well known that P-type ATPase contains one catalytic site per protomer (or monomer). All the sites can be phosphorylated by $P_i + Mg^{2+}$ and are able to bind ATP without the presence of Mg^{2+} (*9*) in H/K-ATPase. However the maximum amount of phosphoenzyme (EP) formed from ATP + Mg^{2+} is exactly half the amount obtained from $P_i + Mg^{2+}$, 0.5 mol of EP/mol of α-chain (*9*, *10*). We previously showed that each 0.5 mol of EP and EATP/mol of α-chain are simultaneously present in the presence of high concentrations of ATP. This suggests that the liberation of 2 mol of P_i from 1 mol of EP in one subunit and that from 1 mol of EATP in the other, constitutes the rate-determining step for ATP hydrolysis in each subunit in the presence of high concentrations of ATP (*10*). Actually, a number of experiments have demonstrated the existence of oligomeric assem-

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blies (*9–13*) in the case of H/K-ATPase. The oligomeric properties of Na/K- and Ca-ATPase have been also reported from structural and functional points of view (*14–17*). On the other hand, the detergent-solubilized αβ-protomer of Na/K-ATPase and the monomer of Ca-ATPase are reported to retain their ability to catalyze ATP hydrolysis (*18–23*). The elucidation of the monomeric crystal structures of SERCA1a (*24*, *25*) has led to a better understanding of the mechanism of P-type ATPases, such as the molecular events induced by high affinity ATP binding in the presence of Ca^{2+} and Mg^{2+} (*26*, *27*). However, the role of low affinity ATP binding to the P-type ATPases in the presence of physiological concentrations of ATP remains unknown. This would be related to the oligomeric properties of the enzyme and cation transport across the membrane (*8*, *10*). Thus, an investigation of the oligomeric properties of the enzyme and its function are highly desirable.

We recently showed that the proportion of *n*-octyl glucoside (OG)–solubilized tetraprotomer of fluorescein 5′-isothiocyanate (FITC)-modified H/K-ATPase (FITC-H/K-ATPase), as detected by a single-molecule detection technique using total internal reflection fluorescence microscopy (TIRFM), is close to the proportion of the OG-solubilized specific activity of H/K-ATPase in the supernatant, calculated from the relative specific activity of unmodified H/K-ATPase prior to solubilization (*13*). High performance gel chromatography (HPGC) also indicated a similar proportion of tetraprotomer in the solubilized H/K-ATPase (*13*). These data, the first observation of a tetraprotomeric FITC-H/K-ATPase, provide qualitative support for the importance of a tetraprotomeric structure in H/K-ATPase reactions. However, a direct and more quantitative comparison between the amount of soluble oligomeric species in FITC-H/K-ATPase by TIRFM and soluble H/K-ATPase activity is not available. FITC treatment nearly completely abolishes H/K-ATPase activity (*28*, *29*), which is inevitable for the direct detection of a tetraprotomer by TIRFM. Paranitrophenyl phosphate (pNPP), a less bulky substrate than ATP, is hydrolysed by P_2-type ATPases and the reaction is negligibly affected by FITC treatment (*28–31*). Thus it should be possible to determine the K^+-dependent pNPPase activity of soluble FITC-H/K-ATPase. The presence of tetraprotomeric H/K-ATPases independent of FITC treatment was also unequivocally demonstrated by electron micrographs in octaethylene glycol dodecyl ether ($C_{12}E_8$) solubilized samples (*13*). However, solubilization by $C_{12}E_8$ in the absence of K^+ strongly reduces the H/K-ATPase activity to less than 2% that of the membrane enzyme (*13*), but the enzyme appears to be relatively stable in the presence of K^+ (*32*). In order to determine the correlation between oligomeric assembly and the activity of solubilized gastric H/K-ATPase directly, we investigated (i) the effect of monovalent cations on the stability of $C_{12}E_8$-solubilized H/K-ATPases and followed the activity of K^+-dependent pNPPase (K-pNPPase); (ii) the population of soluble oligomeric species in FITC-H/K-ATPase by TIRFM; and (iii) the activities of K-pNPPase and H/K-ATPase in soluble unmodified H/K-ATPase.

EXPERIMENTAL PROCEDURES

Materials—$C_{12}E_8$ was purchased from Nikko Chemical Co. OG was purchased from Wako Chemical Co. FITC was purchased from Molecular Probes. All other chemicals were of the highest grade available.

Enzyme Preparation and Chemical Modification—Methods for the preparation of vesicles that contain pig gastric H/K-ATPase and their further purification by SDS have been described in previous reports (*33*, *34*). The purified membrane-bound H/K-ATPase preparations were stored in 0.25 M sucrose solutions containing 0.5 mM EGTA/Tris (pH 7.4) at –80°C until used. The specific activity of the purified enzyme was approximately 380–450 μmol P_i/mg/h in a buffer containing 40 mM HEPES/Tris (pH 7.0), 10 mM KCl, 2 mM $MgCl_2$, 25 mM sucrose, 0.1 mM EGTA/Tris and 2 mM ATP/Tris at 37°C. To modify H/K-ATPase with FITC (*13*), purified membrane-bound H/K-ATPase preparations (0.5 mg/ml) were incubated in 1 mM EDTA, 100 mM Tris/HCl (pH 9.2), 0.25 M sucrose and 10 μM of FITC/DMSO at 25°C for 30 min. The modification was terminated by the addition of 1 mM β-mercaptoethanol and the samples were washed twice with 10 mM HEPES/Tris (pH 7.0), 1 mM EDTA and 0.25 M sucrose. The residual ATPase activity of the FITC modified ATPase preparation was 0.5–1.5% that of the unmodified preparation without any detectable loss in K-pNPPase activity. The level of phosphoenzyme (EP) from ATP was also determined to be less than 1% of the unmodified preparation. The amount of bound FITC was determined colorimetrically after solubilization in SDS, to be 4.9 nmol/mg, and the stoichiometry of FITC labeling was calculated to be the same as in our previous report (*13*), *i.e.*, 1 mol of FITC probe/mol of α-chain.

Solubilization of the Membrane Bound Enzyme—For solubilization with $C_{12}E_8$, the purified membrane-bound H/K-ATPase (2 mg/ml) was incubated in medium consisting of 2 mg/ml $C_{12}E_8$, 0.25 M sucrose, 20 mM MES/Tris (pH 5.5) and 0–0.25 M CH_3COOK or 0.1 M salts as indicated in Fig. 1B, at 0°C for 5 min in a final volume of 50 μl (*32*, *35*). To separate the α- and β-chains completely with SDS, the purified membrane-bound H/K-ATPase (2 mg/ml) was incubated in 10 mg/ml SDS, 0.1 M CH_3COONa, 0.25 M sucrose and 20 mM MES/Tris (pH 5.5) at room temperature for 5 min in a final volume of 50 μl. The solubilized samples were centrifuged at 100,000 rpm for 10 min (Optima TL, 100.2 rotor, Beckman), and the supernatant was collected and used as the solubilized H/K-ATPase preparation.

Measurements of Enzymatic Activity and the Amount of Phosphoenzyme—The H/K-ATPase activity of the solubilized H/K-ATPase was measured at 25°C in 40 μl of reaction medium containing 0.1–0.5 μg of solubilized enzyme protein, 100 mM KCl, 20 mM MES/Tris (pH 5.5), 2 mM $MgCl_2$ and 2 mM ATP/Tris in the presence or absence of a specific inhibitor of H/K-ATPase, SCH28080, at a concentration of 10 μM. The reaction was terminated by adding 40 μl of 12% SDS. Inorganic phosphate was measured colorimetrically after reaction with ammonium molybdate (*36*). The K-pNPPase activity was analyzed at 25°C in 40 μl of a reaction medium containing 0.1–0.5 μg of solubilized enzyme protein, 100 mM KCl, 20 mM MES/Tris (pH 5.5), 20 mM $MgCl_2$ and 40 mM pNPP/Tris in the

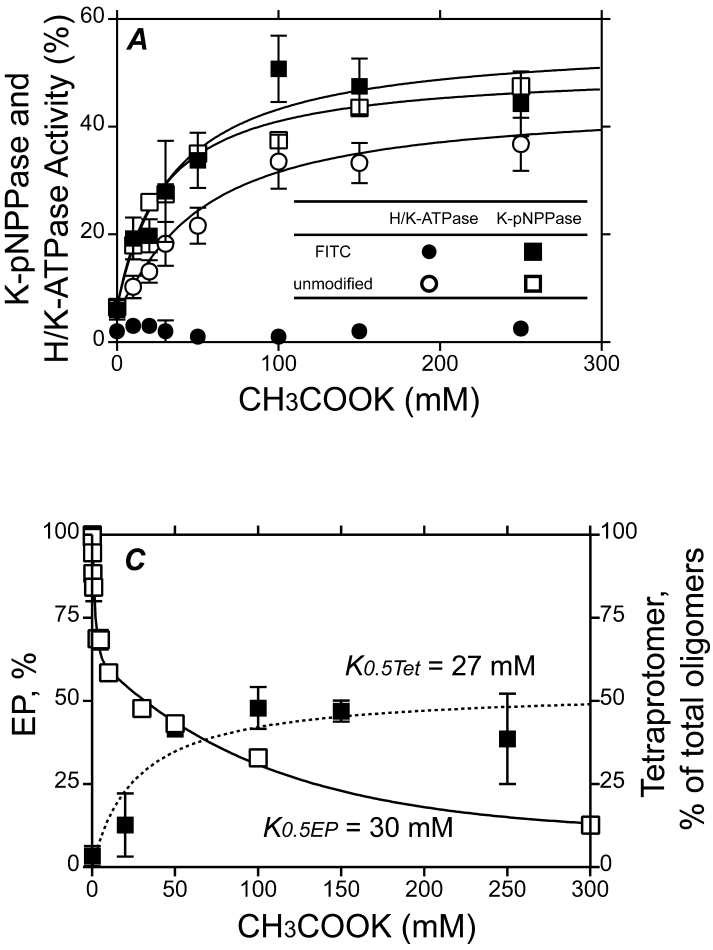

Fig. 1. **Potassium acetate concentration dependence of the activities of H/K-ATPase and K-pNPPase of $C_{12}E_8$-solubilized H/K-ATPase modified with or without FITC.** (A) Membrane-bound H/K-ATPase without FITC treatment (open symbols) or FITC-H/K-ATPase (closed symbols) were solubilized with $C_{12}E_8$ in the presence of the indicated concentrations of CH_3COOK, and the H/K-ATPase (circles) and K-pNPPase (squares) activities of the resulting supernatant were assayed (see "EXPERIMENTAL PROCEDURES"). The specific activities of each sample, except for the H/K-ATPase activity of FITC-H/K-ATPase (closed circles), were calculated as the percentage of the specific activity of membrane-bound enzyme preparations. The ATPase activity of unmodified membrane-bound H/K-ATPase was set as 100% in the case of the H/K-ATPase activity of FITC-H/K-ATPase. (B) The K-pNPPase activities of FITC-H/K-ATPase solubilized with $C_{12}E_8$ in the absence (none) or presence of 100 mM CH_3COOK, KCl, RbCl, LiCl, NaCl or CholineCl. Values are plotted as percentages of the K-pNPPase activity of membrane-bound FITC-H/K-ATPase. (C) K^+ concentration dependence on the amount of phosphoenzyme (open squares) of membrane bound H/K-ATPase at the steady state. The values plotted are the percentages of the amount of EP in the absence of K^+. The population of tetraprotomer (closed squares) in FITC-H/K-ATPase solubilized with $C_{12}E_8$ in the presence of 0, 20, 50, 100, 150 and 250 mM CH_3COOK are also plotted for comparison (see Fig. 3 and "EXPERIMENTAL PROCEDURES" for detail).

presence or absence of 10 μM SCH28080. ATP and pNPP hydrolysis at 25°C were linear up to 30 min. The specific activities of the detergent-solubilized preparations were calculated. The relative specific activities are plotted as the percentage of membrane-bound enzyme in the figures based on at least three independent experiments. The specific activities of H/K-ATPase and K-pNPPase for the purified membrane-bound enzyme were 125 ± 7.1 and 88 ± 4.3 μmol of P_i/mg protein/h, respectively. In all cases, the soluble enzyme was maintained on ice and assayed at 25°C within 3 h after preparation. The amount of phosphoenzyme (EP) was determined as described in Ref. *13*. In a typical run, the membrane-bound H/K-ATPase preparation (0.5 mg/ml) was incubated in a buffer containing 50 mM MES/Tris (pH 5.5), 0.25 M sucrose, 2 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP and 0–300 mM CH_3COOK for 10 s at 0°C in a final volume of 0.1 ml. The reaction was terminated by the addition of 10% trichloroacetic acid. The acid-denatured enzyme was collected, and the incorporated ^{32}P was used to determine the amount of EP.

Single Molecule Imaging—For single-molecule imaging, a flow cell was constructed from two coverslips (bottom, 24 × 36 mm^2; top 18 × 18 mm^2) separated by two 50 μm spacers (*37*). Samples, solubilized by SDS or $C_{12}E_8$, were diluted to approximately 10 pM with buffers containing 0.1 M CH_3COOK, 0.25 M sucrose, 1 mM β-mercaptoethanol and 20 mM MES/Tris (pH 5.5). After a 5 min incubation at room temperature, the flow cell was perfused with buffer containing 0.1 M CH_3COOK, 0.25 M sucrose, 1 mM β-mercaptoethanol and 20 mM Tris/HCl (pH 8.5). An Olympus total internal reflection system was used for the single-molecule observation as described previously (*13*), except that fine adjustments in the optical path and camera setup were made at an S/N ratio

of approximately 1.5. The fluorescence intensities of independent spots with stepwise photobleaching were measured as a 5 video-frame average just after laser illumination. The baseline intensity (the average intensity of 150 video frames collected 15 seconds after laser illumination) was set as zero. The fluorescence intensities of one to four dye molecules were in the linear range of the CCD camera.

To consider the effect of background intensity, the intensities of autofluorescence of the empty space were examined. The mean intensity of the empty space (background) was 1.9–4.7 arbitrary units. Taking the background intensity into account, the histograms for fluorescence intensity were fitted to Gaussian curves; $\Sigma C_i \times \exp[-(x - iM + M_b)^2/2i\sigma^2]$, where i (=1, 2, 3, 4) is the number of Gaussian curves, C_i, M, M_b and σ are the fitting parameters and x is the fluorescence intensity. All fittings were performed using the Prism® (v.2.01, GraphPad Software) software program.

RESULTS

K^+ Dependent Increase of $C_{12}E_8$-Solubilized Enzymatic Activity—Solubilization of pig gastric H/K-ATPase with $C_{12}E_8$ nearly completely abolished both the H/K-ATPase and K-pNPPase activities (*13*). However, it has been reported that when ATP or K^+ is added to the enzyme during the $C_{12}E_8$-solubilization, both activities are retained (*32*, *38*). When unmodified H/K-ATPase was solubilized at pH 5.5 with $C_{12}E_8$ in the absence of potassium acetate, around 5% of the H/K-ATPase and K-pNPPase activities retained (Fig. 1A, open circles and squares) with 60 to 70% of the protein detected in the supernatant. However, these activities in the supernatant (Fig. 1A, open circles and squares) increased with increasing concentrations of potassium acetate to give $K_{0.5}$ values of 40 mM and 28 mM, respectively, without any significant increase in the amount of protein solubilized (data not shown). The maximum extents of the specific activities calculated as a percentage of that for membrane-bound H/K-ATPase (V_{max}) were 42% for H/K-ATPase activity and 51% for K-pNPPase activity. To examine the oligomeric species in the solubilized H/K-ATPase by TIRFM, H/K-ATPase was modified with FITC, which had little effect on K-pNPPase activity but nearly completely inhibited H/K-ATPase activity. The K-pNPPase activity in the supernatant of $C_{12}E_8$-solubilized FITC-H/K-ATPase also increased with increasing concentrations of potassium acetate (Fig. 1A, closed squares, $K_{0.5}$ = 35 mM and V_{max} = 56%). These data suggest that potassium acetate has a similar protective effect on the activities of $C_{12}E_8$-solubilized H/K-ATPase, and that this effect is independent of FITC treatment.

To investigate the specificity of the protective effect of potassium acetate during $C_{12}E_8$-solubilization, potassium acetate was replaced with various other salts. The results clearly show that potassium acetate can be replaced by chloride salts of K^+ or Rb^+ (a K^+ congener), but not by NaCl, LiCl or CholineCl (Fig. 1B). This suggests that the binding of the transporting ligands (K^+ or Rb^+) to the enzyme may be related to the stability of the enzyme activity during solubilization by $C_{12}E_8$. It is known that the apparent K_m of K^+ for the H/K-ATPase reaction is in the mM concentration range, which is lower by one order of magnitude than those required for the stabilization of the enzyme during solubilization with $C_{12}E_8$ (Fig. 1A). To investigate this point, the K^+-dependent reduction of the phosphoenzyme formed from ATP was determined (Fig. 1C). The addition of K^+ showed high and low affinity effects on the amount of phosphoenzyme with an overall $K_{0.5EP}$ value of 30 mM. This value is also close to the concentration of K^+ required, 28–40 mM, to protect the activities of H/K-ATPase and K-pNPPase during solubilization with $C_{12}E_8$ (Fig. 1A) and the concentration for the increase in the population of tetraprotomer in $C_{12}E_8$-solubilized FITC-H/K-ATPase (Fig. 1C, closed squares, see also Fig. 3D) as described later. The results show that the K^+-bound enzyme was stable against $C_{12}E_8$-solubilization regardless of whether or not it was modified with FITC. This allowed us to investigate the relationship between the population of oligomeric species and the K-pNPPase activity of the solubilized FITC-H/K-ATPase, and to relate the oligomeric assembly to the activities of both K-pNPPase and H/K-ATPase in the solublized H/K-ATPase.

Single-Molecule(s) Observation of FITC-H/K-ATPase in Solution—The FITC-H/K-ATPase was solubilized in the presence of various concentrations of potassium acetate or other salts, and analyzed by TIRFM. The fluorescence intensity of FITC-H/K-ATPase just after laser illumination has been shown to be an integral multiple of the single fluorophore (*13*, *39*). The binding stoichiometry of the FITC probe to the enzyme is approximately 1, as has already been reported (*13*, *28*, *29*). We have also shown that the spots observed in TIRFM exhibit stepwise photobleaching, and that initial fluorescence intensity and the steps are well correlated (*13*). The fluorescence intensities of one to four FITC molecules were in the linear range of the detection system. Thus, it becomes possible to estimate the number of protomeric molecules that are assembled from the fluorescence intensity of each spot. Figure 2 shows the distribution of the initial fluorescence intensities of FITC-H/K-ATPase solubilized with $C_{12}E_8$. To completely dissociate the (αβ)-protomer of H/K-ATPase into α- and β-chains, FITC-H/K-ATPase was also solubilized with SDS (Fig. 2A). A typical single-step photobleaching of the FITC probe for a single molecule (*13*) was observed in almost every spot of the completely dissociated FITC-labeled α-chain that had been solubilized with SDS. The initial intensity of each fluorescent spot gave a single peak that could be fitted to a single Gaussian distribution with a mean intensity of 13.4 AU (Fig. 2A), which include background intensity of the empty space (2.2 AU). Thus, each spot represents images of a single molecule of FITC-modified α-chain with a mean intensity of 11.2 AU.

The initial fluorescence intensities of the $C_{12}E_8$-solubilized FITC-H/K-ATPase prepared in the presence of various concentrations of potassium acetate were distributed in a quantized manner (Fig. 2, B–D). These quantized distributions were fitted well to a four-component Gaussian function with the background (see "EXPERIMENTAL PROCEDURES"). The lowest peak intensity of around 14 AU corresponds to the protomeric molecule, and the value is similar to the mean fluorescence intensity of SDS-solubilized FITC-H/K-ATPase (13.4 AU, Fig. 2A).

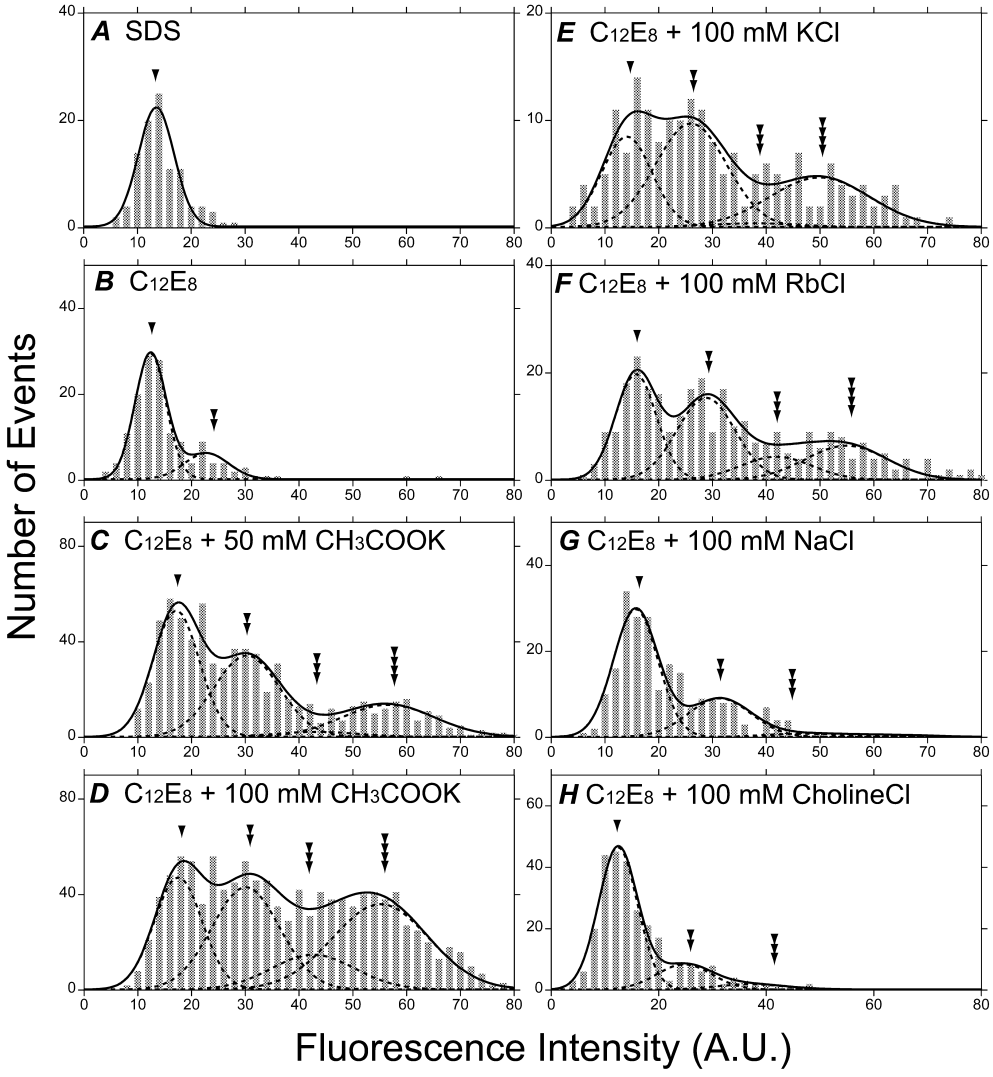

Fig. 2. **Histograms of the fluorescence intensity of $C_{12}E_8$-solubilized FITC-H/K-ATPase observed by TIRFM.** (A) A histogram of SDS-solubilized FITC-H/K-ATPase is shown as the standard fluorescence intensity of a single-molecule. The distribution was fitted to a single component Gaussian function (solid line) with a mean intensity of 13.4 arbitrary units (AU) including the empty space background intensity of 2.2 (see "EXPERIMENTAL PROCEDURES" for details). Histograms for the initial fluorescence intensity of FITC-H/K-ATPase solubilized with $C_{12}E_8$ in the presence of 0 (B), 50 (C) and 100 mM (D) CH_3COOK are shown. FITC-H/K-ATPase was also solubilized by treatment with $C_{12}E_8$ in the presence of 100 mM KCl (E), RbCl (F), CholineCl (G) or NaCl (H), and the intensity distribution evaluated in each sample. Data were fitted to four-component Gaussian curves (see "EXPERIMENTAL PROCEDURES"). The solid lines indicate the sum of one to four Gaussian components fitted to the data and the dotted lines are each individual Gaussian curves. Single, double, triple and quadruple arrowheads indicate the peak fluorescence intensity of one, two, three and four-dye molecules, respectively. The lowest peak intensities distributed around 12.3–17.3 AU, which includes background intensities of 1.9–4.7 AU. The estimated fluorescence intensities responsible for a single dye molecule are distributed from 10.4 to 12.6 AU, which is similar to the estimated mean intensity of SDS-solubilized α-chain (A, 11.2 AU).

The distribution of the initial fluorescence intensities of $C_{12}E_8$-solubilized FITC-H/K-ATPase in the absence of CH_3COOK included only two peaks (Fig. 2B). The fluorescence intensity of the major peak (Fig. 2B, single arrowhead) was indistinguishable from that of SDS-solubilized FITC-H/K-ATPase. The mean intensity of the other was twice as large as the major peak, and can be attributed to the presence of diprotomeric molecules (Fig. 2B, double arrowhead). The distributions of the fluorescence intensities of $C_{12}E_8$-solubilized FITC-H/K-ATPase prepared with 50 and 100 mM CH_3COOK (Fig. 2C and D, respectively) appeared to be composed of four peaks, which are responsible for a 1-, 2-, 3- and 4-fold increase in the basic fluorescence intensities. These peaks with 1, 2, 3 and 4 units can be attributed to the presence of protomer, diprotomer, triprotomer and tetraprotomer, respectively. The tetraprotomer population (Fig. 3, C and D, quadruple arrowheads) clearly increased with increasing concentrations of CH_3COOK. The presence of 100 mM KCl (Fig. 2E) or RbCl (Fig. 2F) during solubilization had a similar effect on the distribution of the initial fluorescence intensity as the presence of 100 mM CH_3COOK (Fig. 2E). Neither NaCl (Fig. 2G) nor CholineCl (Fig. 2H) had any significant enhancement in the amount of tetraprotomer.

Correlations between Enzymatic Activity and Oligomeric Structure—To investigate the relationships between activity and the oligomeric assembly of $C_{12}E_8$-solubilized FITC-H/K-ATPase, the population of each oligomeric species of the enzyme in the presence of 0, 20, 50, 100, 150 and 250 mM potassium acetate were plotted against the K-pNPPase activity of $C_{12}E_8$-solubilized FITC-H/K-ATPase (Fig. 3, closed circles and lines). Because the histograms shown in Fig. 2 show the plotted data for the number of observed fluorescence particles against the fluorescence intensity of each spot, the areas of estimated Gaussian curves responsible for the diprotomer, triprotomer and tetraprotomer were multiplied by 2, 3 and 4, respectively to estimate the relative populations of the oligomeric components. The relative proportion of protomer, diprotomer, triprotomer and tetraprotomer thus calculated with increasing concentrations of CH_3COOK

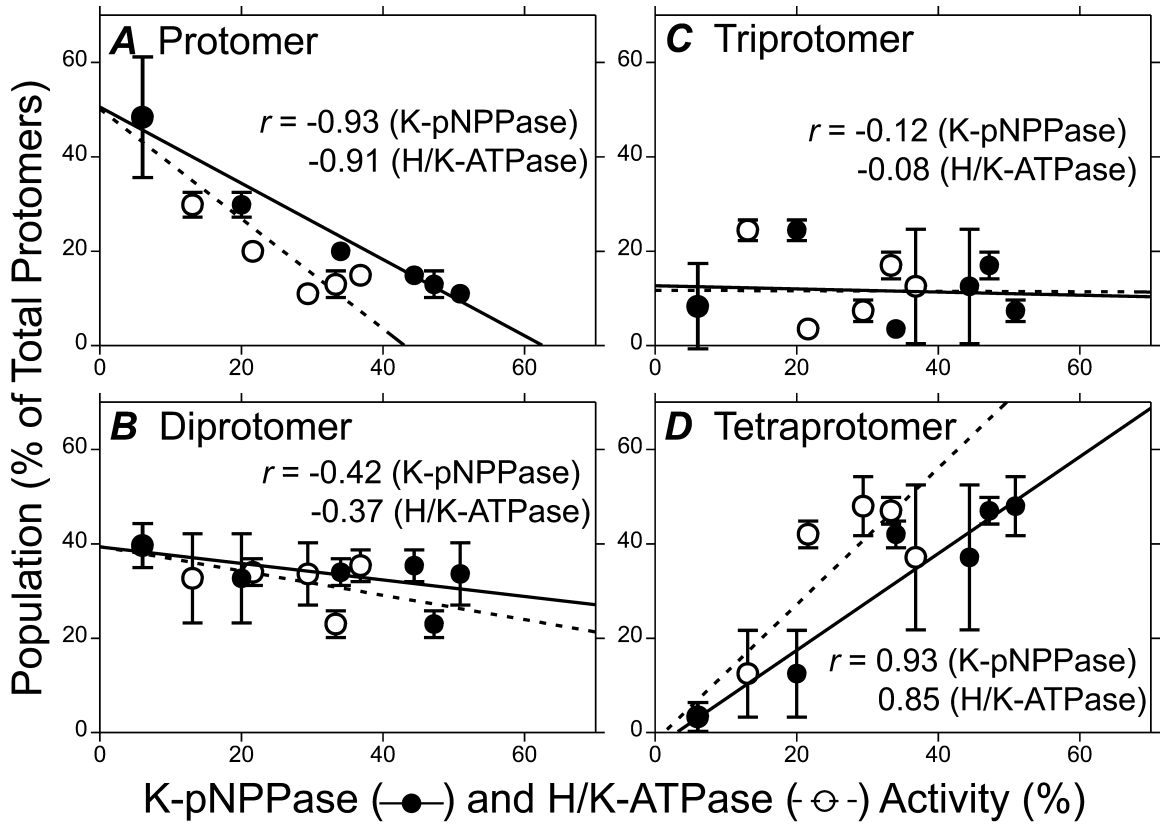

Fig. 3. **Correlation between the population of oligomeric species and the enzyme activities in $C_{12}E_8$-solubilized FITC-H/K-ATPase.** The population of protomer (A), diprotomer (B), triprotomer (C) and tetraprotomer (D) in FITC-H/K-ATPase solubilized with $C_{12}E_8$ in the presence of 0, 20, 50, 100, 150, 250 mM CH_3COOK were plotted as a function of the K-pNPPase activity of the corresponding FITC-H/K-ATPase samples (closed circles, lines), and as a function of the H/K-ATPase activity of the corresponding unmodified H/K-ATPase (open circles, broken lines) indicated in Fig. 1. Correlation coefficients, abbreviated as *r*, are shown in the figure. The population of each oligomer species was calculated as the percentage of total protomer, namely, [area of Gaussian curve (Fig. 2)] × [number of protomer in oligomer species] in the solubilized FITC-H/K-ATPase samples.

as typical examples were 63, 37, 0 and 0% with 0 mM CH_3COOK (Fig. 2B); 28, 26, 26 and 19% with 20 mM CH_3COOK (not shown); 20, 36, 4 and 40% with 50 mM CH_3COOK (Fig. 2C); and 9, 23, 14 and 54% with 100 mM CH_3COOK (Fig. 2D). The relative population of the tetraprotomer and the residual K-pNPPase activity obtained in at least three independent experiments, as shown in Fig. 1, were nearly the same within experimental error for each concentration of CH_3COOK; namely, 0% and 6% ± 2% with 0 mM CH_3COOK; 19% and 20 ± 3% with 20 mM CH_3COOK; 40% and 34 ± 5% with 50 mM CH_3COOK; and 54% and 51 ± 6% with 100 mM CH_3COOK. The correlation coefficient between the relative population of the tetraprotomer and the relative activities was 0.93 ($P < 0.01$) which is rather close to 1, while the other coefficients were far from 1 and negative: –0.93 (Protomer, $P < 0.01$), –0.42 (Diprotomer, $P < 0.2$) or close to zero, –0.12 (Triprotomer, $P < 0.7$). These data indicate a good correlation between the amount of tetraprotomer and activity. One may ask whether the larger error bars due to a 4-fold multiplication of the errors in Fig. 3D seriously effects our conclusions. This possibility was considered and rejected because nearly the same correlation coefficients were obtained when the correlation coefficient of the relative amount of tetraprotomer with the lowest and the highest values and activities were calculated (0.95 and 0.93; nearly the same as the values presented above). In addition, the slope of the line in Fig. 3D, (1.03) suggests that the population of tetraprotomer in solubilized sample is close to that of the pNPPase activity. The strong positive correlation for the tetraprotomer (Fig. 3D) and the negative correlation for the protomer (Fig. 3A) with little correlation for either the diprotomer or triprotomer (Fig. 3, B and C) suggest that K-pNPPase activity, catalyzed by the K^+-bound enzyme form, requires tetraprotomeric assembly of the $C_{12}E_8$-solubilized FITC-H/K-ATPase.

DISCUSSION

The FITC-modification of P-type ATPases, such as gastric H/K-ATPase (*29*), kidney Na/K-ATPase (*30*) and sarcoplasmic Ca-ATPase (*31*) is known to be one of the most specific chemical modifications resulting in the modification of a specific Lys-residue in the ATP binding pocket (*24*). The FITC-modified enzymes lose only their high affinity ATP binding, which results in no phosphorylation by ATP in the forward reaction. However, the ATP utilized in the phosphorylation of the FITC-modified enzyme can be replaced by less bulky substrates than ATP such as acetyl phosphate and *p*-nitrophenyl phosphate to form the phosphoenzyme by the forward reac-

tion (*9*, *40*) and FITC-modified enzyme retains its K^+-dependent p-NPPase activity almost completely. Thus we used a fluorescent FITC-modified enzyme to follow the relationship between the oligomeric assembly of the protein and the enzymatic activity. The amount of oligomeric species in the solubilized FITC-H/K-ATPase was estimated by a single-molecule detection technique (*13*). As a control, the relationship between the oligomeric assembly and activities of both the H/K-ATPase and K-pNPPase of the unmodified enzyme were also analyzed as described below. Solubilization of the enzyme strongly reduced its K-pNPPase activity, and the addition of potassium acetate induced an increase in K-pNPPase activity with an increase in the amount of the tetraprotomer of FITC-H/K-ATPase. The correlation coefficient between the proportion of tetraprotomer and the proportion of the K-pNPPase activity within the same FITC-H/K-ATPase preparation was estimated to be 0.93. The protective effect of K^+ on enzyme activity upon $C_{12}E_8$-solubilization was independent of FITC modification. Thus, we also plotted the population of oligomeric species in the solubilized FITC-H/K-ATPase against the H/K-ATPase and the K-pNPPase activities of unmodified H/K-ATPase that had been solubilized in the same manner as FITC-H/K-ATPase. A similar positive correlation between the increase in the amount of tetraprotomer and increases in the activities of both H/K-ATPase (Fig. 3D, open circles and broken line, $r = 0.86$, $P < 0.01$) and K-pNPPase ($r = 0.85$, $P < 0.01$, not shown in figure) was obtained, and negative correlations (Fig. 3A and B, open circles and broken lines) between increases in the amounts of protomer and diprotomer and decreases in the both activities of the solubilized unmodified H/K-ATPase were also observed as in the case of the correlation within the same FITC-H/K-ATPase (closed circles and lines in Fig. 3D, A and B). These data strongly suggest that the tetraprotomer in the $C_{12}E_8$-solubilized unmodified H/K-ATPase also retained both H/K-ATPase and K-pNPPase activities.

The tetraprotomer of H/K-ATPase was isolated under $C_{12}E_8$- or OG-solubilized conditions by HPGC, not under aggregated conditions, as previously reported (*13*). Active tetraprotomeric species in the $C_{12}E_8$-solubilized FITC-H/K-ATPase require the presence of K^+ or its congeners and the presence of LiCl, NaCl or choline chloride strongly reduced the amount of tetraprotomer present (Figs. 1 and 2). The K^+-dependent increase in the population of tetraprotomer was accompanied by a decrease in the population of protomers and diprotomers, while the population of triprotomer was maintained at a lower constant level (Fig. 3). These data suggest that the successful $C_{12}E_8$-solubilization of H/K-ATPase in the presence K^+ (*35*) is due to the preservation of a tetraprotomeric assembly from the inactivation due to the dissociation into two diprotomers and then to four protomers in the $C_{12}E_8$-solubilized conditions. The data also exclude the possibility that the presence of a tetraprotomer in $C_{12}E_8$-solubilized FITC-H/K-ATPase was the result of non-specific protein aggregation due to the dilution of the detergent concentration in the TIRFM analysis.

The binding of the physiological ligand K^+ to H/K-ATPase induced the transition of the phosphoenzyme to a K^+-bound enzyme (Fig. 1C, open squares), as detected by a change in the microenvironment of Cys-241 of the α-chain (*41*). The present data show that K^+ induces changes, not only in the tertiary structure, but also in the quaternary structure as detected by the presence of a K^+-dependent active and soluble tetraprotomer under $C_{12}E_8$-solubilized conditions (Fig. 2). When the population of tetraprotomer in $C_{12}E_8$-solubilized FITC-H/K-ATPase was plotted as a function of K^+ concentration as shown in Fig. 1C (closed squares), a $K_{0.5}$ value similar to the transition was obtained. This indicates that the conformational state of H/K-ATPase in the lipid bilayer is related to the stability of the tetraprotomer to $C_{12}E_8$-solubilization. Removal of the boundary lipid as the result of $C_{12}E_8$-solubilization has been reported to induce the inactivation of H/K-ATPase activity (*12*), suggesting that phospholipids are related to the assembly of the K^+-stabilized soluble tetraprotomeric form of H/K-ATPase. The presence of phosphatidylserine is required to stabilize the tetraprotomer in $C_{12}E_8$-solubilized Na/K-ATPase and the population of tetraprotomer increased with added K^+ and decreased with added Na^+ (*35*, *42*, *43*). Rb^+ occlusion in the cation-binding site of H/K-ATPase has been reported to stabilize the tertiary structure of the enzyme (*44*). Such a ligand-dependent conformational change may induce a change in the environment around the interface between the protomers in the tetraprotomeric enzyme, resulting in a change in detergent accessibility for the hydrophobic surface and boundary lipids. Both the K^+-dependent increases in activities and the population of tetraprotomer suggest that K^+ binding or occlusion stabilizes the tetraprotomeric structure protecting it from attack by $C_{12}E_8$. The tertiary folding of the first tetramerization domain of the Kv channels has been shown to couple to their tetramer formation in their biogenetic stage (*45*). Such coupling between tertiary and quaternary structures may be a common feature of oligomeric proteins.

Treatment of H/K-ATPase with OG induced the solubilization of both H/K-ATPase activity and K-pNPPase activity in the unmodified H/K-ATPase (*38*) as well as the tetraprotomer in FITC-H/K-ATPase (*13*). However, OG-solubilized FITC-H/K-ATPase was nearly completely devoid of K-pNPPase activity. These data suggest that high affinity ATP binding is required to preserve catalytic activity during solubilization with OG (*37*). The present data also indicate that the role of K^+ in the production of active $C_{12}E_8$-solubilized H/K-ATPase (*32*, *38*) is to protect tetraprotomeric assembly, which is required not only for K-pNPPase activity but also for H/K-dependent ATP hydrolysis accompanying the sequential appearance of various reaction intermediates (*1*, *7*, *8*). Although the simultaneous presence of EP:EATP, half site phosphorylation and the reduction of EP breakdown by bound ATP by membrane H/K-ATPase have already been demonstrated (*10*), to clarify the reason that teraprotomeric assembly is required is key to our understanding of the mechanism of ion transport across membranes.

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