Fluorescence Energy Transfer Studies of Purified Erythrocyte Ca\(^{2+}\)-ATPase

Ca\(^{2+}\)-REGULATED ACTIVATION BY OLIGOMERIZATION

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Fluorescence resonance energy transfer has been used to study oligomerization of the purified erythrocyte Ca\(^{2+}\)-ATPase. The energy transfer efficiency has been measured at different enzyme concentrations, from fluorescein 5'-isothiocyanate attached to one enzyme molecule to eosin 5-maleimide or tetramethylrhodamine 5-isothiocyanate attached to another enzyme molecule. The energy transfer efficiency showed a sigmoidal dependence on enzyme concentration and was half-maximal at 10–12 nM enzyme; this dependence on enzyme concentration closely resembled previously demonstrated dependence of Ca\(^{2+}\)-ATPase activity and polarization of the fluorescein 5'-isothiocyanate enzyme (Kosk-Kosicka, D., and Bzdega, T. (1988) J. Biol. Chem. 263, 18184–18189). Thus, the three independent methods establish that enzyme concentration-dependent oligomerization is a mechanism of activation of the erythrocyte Ca\(^{2+}\)-ATPase.

Further energy transfer studies demonstrated that enzyme oligomerization required calcium. This calcium dependence was characterized by high affinity (half-maximal energy transfer at pCa 7.15) and cooperativity (Hill coefficient of 2.36), being very similar in both respects to the Ca\(^{2+}\) dependence of the Ca\(^{2+}\)-ATPase activity. The data indicated that the oligomerization process produced a highly cooperative, Ca\(^{2+}\)-regulated activation of the enzyme at physiologically relevant Ca\(^{2+}\) concentrations. These studies show that the Ca\(^{2+}\)-ATPase can be fully activated by a Ca\(^{2+}\)-dependent oligomerization mechanism, which is independent of the previously described activation by calmodulin. We propose two pathways for the activation of the Ca\(^{2+}\)-ATPase, taking into account the interdependencies between the Ca\(^{2+}\), calmodulin, and enzyme concentrations.

Erythrocyte Ca\(^{2+}\)-ATPase is a representative of plasma membrane Ca\(^{2+}\) pumps which are modulated by calmodulin (1, 2). One calmodulin binding site has been identified in the C terminus of the enzyme, in agreement with a 1:1 stoichiometry shown for calmodulin binding and activation of this enzyme (3–5). The mechanism of the regulation is not well understood.

Recently we proposed another mechanism of activation of the erythrocyte Ca\(^{2+}\)-ATPase, by self-association in an enzyme concentration-dependent manner (5). The hypothesis, based upon observation of Ca\(^{2+}\)-ATPase activity in the presence and absence of calmodulin, received support through observations of enzyme concentration-dependent changes in polarization (5) and differences in calmodulin binding to the putative monomers and oligomers (6).

In this study, we have sought to develop a direct physical method of measuring oligomerization, totally independent of activity measurements, which would allow us to test both the oligomerization hypothesis and to establish factors which promote oligomerization. For these purposes we have employed fluorescence resonance energy transfer (FRET) between Ca\(^{2+}\)-ATPase populations separately labeled with either a fluorescence donor or acceptor. Using this method, we confirm our hypothesis and further show that the oligomerization process requires Ca\(^{2+}\). Part of this study has been presented in preliminary form (7).

MATERIALS AND METHODS

Egg yolk phosphatidycholine (P5763) and CNBr-activated Sepharose 4B were purchased from Sigma; C\(_{12}\)E\(_8\) was from Nikkol, Japan. Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia instructions, as described earlier (8, 9). Purification of Ca\(^{2+}\)-ATPase—The Ca\(^{2+}\)-ATPase was purified from erythrocyte ghosts by calmodulin affinity column chromatography in the presence of the nonionic detergent C\(_{12}\)E\(_8\), as described previously (5, 8, 9). The enzyme was stored at −80 °C in elution buffer containing 10 mM Tris-maleate, pH 7.4, 130 mM KCl, 0.5 mM MgCl\(_2\), 5 mM EGTA, 20% glycerol, 750 μM C\(_{12}\)E\(_8\), 2 mM dithiothreitol, and 0.02% of sonicated suspension of egg yolk phosphatidycholine.

Protein Assay—The concentration of enzyme protein in the eluate was measured by the Lowry method (10) modified according to Bensadoun and Weinstein (11), as well as by the Bio-Rad Protein Micro-assay, based on the Bradford dye-binding procedure (12). Both methods gave similar values within 5% error. Bovine serum albumin was used as a standard. The Ca\(^{2+}\)-ATPase molecular weight of 140,000 determined by gel electrophoresis was used to calculate molar amounts of the protein (9).

Ca\(^{2+}\)-ATPase Activity—Ca\(^{2+}\)-ATPase activity was determined by measurement of inorganic phosphate production, as described previously (5). The assay was performed in a reaction mixture containing 50 mM Tris-maleate, pH 7.4, 130 mM KCl, 8 mM MgCl\(_2\), 3 mM ATP, 1 mM EGTA, and C\(_{12}\)E\(_8\) in concentrations yielding the required free Ca\(^{2+}\). The reaction volume was 100 μl. Appropriate aliquots of the Ca\(^{2+}\)-ATPase in the elution buffer were added to achieve the desired enzyme concentration. The concentration of C\(_{12}\)E\(_8\) was kept constant.

\(^{1}\) The abbreviations used are: FRET, fluorescence resonance energy transfer; C\(_{12}\)E\(_8\), octaethylene glycol mono-n-dodecyl ether; EGTA, [ethylenebis(oxyethylenenitrilo)]etraacetic acid; Tris, tris(hydroxymethyl)aminomethane; FITC, fluorescein 5'-isothiocyanate; EM, eosin 5-maleimide; TRITC, tetramethylrhodamine 5-isothiocyanate.
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at 150 \textmu M. The reaction was started with 3 mM ATP and carried out for up to 30 min at 37 °C. Aliquots were withdrawn at various times for colorimetric inorganic phosphate measurement with malachite green (13). Steady-state velocities were obtained from plots of inorganic phosphate production which were linear with time.

Free Calcium—Free Ca\textsuperscript{2+} concentrations were calculated (14) from total calcium (total Ca\textsuperscript{2+} and EGTA), based on the constants given by Schwartzbach et al. (15) and the pKa values given for EGTA by Blinks et al. (16), taking into account pH, ionic strength, magnesium, and ATP concentrations. Total Ca\textsuperscript{2+} was measured by atomic absorption.

Fluorescence Measurements—Fluorescence excitation and emission spectra of the FITC-Ca\textsuperscript{2+}-ATPase, TRITC-Ca\textsuperscript{2+}-ATPase, and EM-Ca\textsuperscript{2+}-ATPase were recorded with an SLM-8000 spectrophotometer, using excitation and emission slits of 8 nm. Excitation and emission wavelengths used for the three fluoroescently labeled Ca\textsuperscript{2+}-ATPase preparations were 470 and 525, 550 and 570, 530 and 550 nm, respectively. Aliquots of the labeled enzyme, from 5 to 100 \textmu M FITC or 60 \textmu M EM; 1.75 \textmu M FITC or 60 \textmu M TRITC. The unreacted probe was removed by two washings in 5 volumes of a medium containing 10 mM Tris-maleate, pH 7.5, 130 mM KCl, 0.5 mM MgCl\textsubscript{2}, 50 \textmu M CaCl\textsubscript{2}, and 10% glycerol. The labeled Ca\textsuperscript{2+}-ATPase was then purified by our standard procedure described above. The stoichiometry of labeling was measured after the purified enzyme was eluted from the Cam-Sepharose column (for details on enzyme purity, see Ref. 5). €0.0 = 9.6 \times 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1} was used for the TRITC-labeled enzyme, €5.2 = 8.4 \times 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1} for the EM-labeled enzyme (17), and €8.0 = 8 \times 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1} for the FITC-labeled enzyme, in the presence of 0.1% NaOH (18).

Energy Transfer Measurements—The following donor-acceptor pairs were used in energy transfer studies between Ca\textsuperscript{2+}-ATPase molecules: FITC-ATPase (donor)-TRITC-ATPase (acceptor); FITC-ATPase (donor)-EM-ATPase (acceptor). The suitability of the pairs was determined by the overlap (J) between the emission spectrum of the donor and the absorption spectrum of the acceptor. The overlap was characteristic of high values, comparable to the expected distances between fluorophores on different Ca\textsuperscript{2+}-ATPase molecules and to the high extinction coefficient of the donor.

In the course of the study we have labeled erythrocyte membranes with either FITC, TRITC, or EM as described under “Materials and Methods,” and the derivatized Ca\textsuperscript{2+}-ATPase was subsequently purified by our standard procedure (9). The extent of labeling by all three probes saturated at around 1:1 mol of the label/mol of the purified enzyme, coinciding with a complete inhibition of the Ca\textsuperscript{2+}-ATPase activity (not shown). Millimolar ATP protected the enzyme from modification by the lysine-directed probes, FITC and TRITC, in accordance with their expected binding to lysine 601 within the nucleotide binding site (20, 21). ATP did not prevent enzyme derivatization with EM, a sulphhydril-directed probe (17).

Fig. 1C shows fluorescence intensity emission spectra of the FITC-labeled, purified Ca\textsuperscript{2+}-ATPase alone and after addition of an equal amount of the EM-labeled Ca\textsuperscript{2+}-ATPase. Addition of the acceptor-labeled enzyme caused a decrease of the fluorescence intensity of the FITC (donor-labeled enzyme) with a maximum at 525 nm and an increase in the fluorescence emission of the EM with a maximum at 550 nm. Similar changes were observed when TRITC was used as acceptor (not shown). The decrease in donor emission at 525 nm was subsequently used for calculations of FRET because it was an "optical window" free of the acceptor’s emission. At other wavelengths, e.g. 550 nm (maximum emission of EM), an overlap of donor-acceptor spectra obscured the calculations. The following evidence shows that the decrease in FITC emission at 525 nm was caused by specific associations between the donor- and the acceptor-labeled enzyme molecules and not by an artifact due to direct absorption by the acceptor at either the 470 nm excitation or the 525 nm emission wavelengths of the donor. First, the extinction coefficients of the acceptor-labeled enzyme were at least 10-fold lower than

![Fig. 1. Suitability of the FITC-EM and FITC-TRITC pairs for studies on energy transfer between enzyme molecules. A and B show the spectral overlap of donor emission of the FITC-labeled enzyme and acceptor absorption spectra of the two acceptor pairs: FITC-EM and FITC-TRITC. These overlaps were characteristic of high \( R_0 \) values, comparable to the expected distances between fluorophores on different Ca\textsuperscript{2+}-ATPase molecules and to the high extinction coefficient of the donor.](image-url)
that of the donor ($\epsilon_{\text{d}} = 3 \times 10^4 \text{ M}^{-1}$, and $5 \times 10^5 \text{ M}^{-1}$ for the EM- and TRITC-labeled enzyme, respectively). For such small optical densities the influence of the absorption of the acceptor on the donor emission was negligible. Second, the decrease in FITC emission at 525 nm was not observed upon addition of the acceptor-labeled Ca$^{2+}$-ATPase after it was heat-denatured. The sodium dodecyl sulfate-gel electrophoresis showed that the label (EM or TRITC) stayed attached to the enzyme protein.

**Dependence of Fluorescence Energy Transfer Efficiency on Ca$^{2+}$-ATPase Concentration**—The experiment described in Fig. 1C was repeated at different total enzyme concentrations. We quantified the decrease in donor fluorescence observed upon addition of the acceptor-labeled enzyme and constructed curves representing energy transfer efficiency as a function of enzyme concentration. As shown in Fig. 2, a similar pattern was observed with both donor-acceptor pairs used: no measurable energy transfer occurred at low enzyme concentrations, and a maximum was reached at around 20 nM total enzyme. The observation of a plateau at high enzyme concentrations indicated that there was no significant component of FRET that would originate from random collisions of enzyme molecules. Although slightly higher values were obtained with the FITC/TRITC pair than with FITC/EM, the half-maximal energy transfer occurred at very similar enzyme concentrations, between 10 and 12 nM Ca$^{2+}$-ATPase, for both pairs.

The enzyme concentration-dependent increase in energy transfer (Fig. 2) showed a striking resemblance to the previously reported dependence of the Ca$^{2+}$-ATPase activity and fluorescence polarization on enzyme concentration (5). These three data sets are compared in Fig. 3. The very similar dependence shown by the three methods supports the conclusion that the erythrocyte Ca$^{2+}$-ATPase is indeed activated by self-association in an enzyme concentration-dependent manner.

**Ca$^{2+}$ as a Limiting Factor in Energy Transfer and Ca$^{2+}$-ATPase Activity**—When energy transfer between FITC-Ca$^{2+}$-ATPase and EM-Ca$^{2+}$-ATPase molecules was studied at nanomolar Ca$^{2+}$ concentrations, as opposed to micromolar Ca$^{2+}$ used in the preceding experiments, the maximal level of FRET was 2.2% instead of 6.6% (compare * and ○ in Fig. 2). It should be noted that fluorescence emission of the FITC-labeled enzyme alone was not affected by changes in Ca$^{2+}$ concentration (not shown); thus, FRET efficiency is unlikely to be altered by Ca$^{2+}$-induced conformational changes in the enzyme. This significantly smaller energy transfer efficiency indicated that nanomolar Ca$^{2+}$ was a limiting factor in enzyme oligomerization.

Similar dependence on Ca$^{2+}$ was observed in the behavior of enzyme activation (Fig. 4). When enzyme concentration-dependent Ca$^{2+}$-ATPase activity was compared at 17 μM and 50 nM free Ca$^{2+}$, a close resemblance with the curves representing energy transfer efficiency (Fig. 2) was observed in respect to both $K_m$ and maximal values. Half-maximal activation (Fig. 4) as well as half-maximal oligomerization (Fig. 2) occurred at 8–12 nM enzyme. Like the energy transfer efficiency, the Ca$^{2+}$-ATPase activity was also about three times lower at nanomolar Ca$^{2+}$ than at micromolar Ca$^{2+}$. The relationship between Ca$^{2+}$-ATPase activity and FRET was analyzed by a secondary plot described in Fig. 5. At both Ca$^{2+}$ concentrations studied, the activity increased linearly with the increase of oligomerization (measured by FRET) supporting a functional requirement for enzyme protein associations.

**Ca$^{2+}$ Dependence of Enzyme Activation by Oligomerization**—To establish the crucial Ca$^{2+}$ concentration required for oligomerization of the enzyme, energy transfer efficiency was studied over a broad pCa range at 30 nM Ca$^{2+}$-ATPase. Fig. 6
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Several other methods, like sedimentation velocity, sedimentation equilibrium, and gel filtration, proved to be unsuitable. The FRET method has been well established from a theoretical standpoint and was applied widely to many other systems in studies of both inter- and intramolecular energy transfer (19, 21–25).

To study the intermolecular energy transfer, we have modified the Ca\textsuperscript{2+}-ATPase with the given fluorescent donor or acceptor in the enzyme's natural environment in the ghost membrane, and only then purified. The measured energy transfer was shown to originate exclusively from the functional mixed donor-acceptor-labeled Ca\textsuperscript{2+}-ATPase oligomers. The efficiency did not increase further once it reached its maximal level at 20 nM enzyme, indicating that there was no significant FRET component originating from random collisions of enzyme molecules (Fig. 2). Additionally, no energy transfer occurred when the acceptor-labeled Ca\textsuperscript{2+}-ATPase was denatured. The accuracy of the method was also shown by similarity of the half-maximal enzyme concentrations for energy transfer from FITC to the two different acceptors used, EM and TRITC (Fig. 2). Thus, using the FRET method, we have confirmed that the erythrocyte Ca\textsuperscript{2+}-ATPase oligomerizes in an enzyme concentration-dependent manner and further shown that this process leads to Ca\textsuperscript{2+}-ATPase activation (Figs. 2 and 4).

Ca\textsuperscript{2+} Dependence of Ca\textsuperscript{2+}-ATPase Oligomerization—A further strength of the FRET method is that Ca\textsuperscript{2+} dependence of oligomerization could be measured independently of Ca\textsuperscript{2+} dependence of the Ca\textsuperscript{2+}-ATPase activity. The energy transfer efficiency at a given enzyme concentration diminished at lower Ca\textsuperscript{2+} concentration as the amount of oligomeric enzyme was decreased (Figs. 2 and 6). Thus, using the FRET method we have shown, directly that the oligomerization process is Ca\textsuperscript{2+} concentration-dependent (Fig. 6). This Ca\textsuperscript{2+} dependence is characterized by high affinity and cooperativity, being very similar in both respects to the Ca\textsuperscript{2+} dependence of the Ca\textsuperscript{2+}-ATPase activity in the absence of calmodulin, as well as to the Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} binding to the enzyme (8). The high degree of Ca\textsuperscript{2+} cooperativity ($n_K = 2.6$–3.0) can now be explained as a combined effect of both Ca\textsuperscript{2+}-stimulated oligomerization and Ca\textsuperscript{2+}-dependent binding to the two putative Ca\textsuperscript{2+} transport sites. These data indicate that the Ca\textsuperscript{2+}-dependent oligomerization of the enzyme can, in the absence of calmodulin, produce an enzyme conformation that exhibits maximal Ca\textsuperscript{2+}-ATPase activity at physiologically relevant Ca\textsuperscript{2+} concentrations.

The Mechanism of Enzyme Activation—The presented data together with our previous findings show that three factors, Ca\textsuperscript{2+}, calmodulin, and enzyme concentration, influence the equilibrium between monomers and oligomers, and then make respective contributions to the observed Ca\textsuperscript{2+}-ATPase activity. Ca\textsuperscript{2+} is absolutely required for enzyme activation either by calmodulin or by enzyme oligomerization (Figs. 2, 4, and 6 and Refs. 5 and 6). The enzyme concentration affects the extent of equilibration between monomers and oligomers at any given Ca\textsuperscript{2+} concentration (Figs. 2 and 4). Calmodulin activates monomers but not oligomers (5, 8, 9).

The differences in Ca\textsuperscript{2+} dependencies of the two processes suggest that oligomerization may be switched on at a lower Ca\textsuperscript{2+} concentration ($K_C = pC_a 7.15$) than activation by calmodulin ($K_C = pC_a 6.6$). These differences in Ca\textsuperscript{2+} affinity might be physiologically important, assuming that oligomerization occurs in vivo. In fact other studies, radiation inactivation of the erythrocyte membranes and nondenaturing gel analysis of the azido \textsuperscript{151}I-calmodulin-labeled enzyme, support the contention that the Ca\textsuperscript{2+}-ATPase exists as a dimer.
in the membrane in vivo (26–28). Also the enzyme concentration in erythrocyte membrane, 0.1% of total protein, is compatible with high probability of enzyme undergoing oligomerization (1, 2).

A mechanism could be envisaged such that the two pathways, activation by calmodulin or by oligomerization, are activated depending on environmental conditions; among them are the level to which cytosolic Ca\(^{2+}\) has risen, availability of calmodulin for interaction with the Ca\(^{2+}\)-ATPase, or other factors that have been demonstrated to modulate the Ca\(^{2+}\)-ATPase, such as endogenous proteases, phospholipids, or phosphorylation (29–31). The concentration of calmodulin in the cell is probably on the same order (1–5 \(\mu\)M) as the total concentration of its target proteins, such that small changes in its effective concentration might make it unavailable to the Ca\(^{2+}\)-ATPase (32).

Acknowledgments—We thank Dr. James Bishop for valuable discussion and for reviewing the manuscript, and we thank Dr. Robert Steiner of the University of Maryland Baltimore Campus for a generous gift of bovine testes calmodulin used for affinity chromatography in enzyme purification, and for the availability of his spectrofluorometer.

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