Human Erythrocyte Calmodulin

FURTHER CHEMICAL CHARACTERIZATION AND THE SITE OF ITS INTERACTION WITH THE MEMBRANE

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Human erythrocyte and bovine brain calmodulins were indistinguishable by tryptic peptide mapping, indicating that the primary sequence of the two proteins is either very similar or identical. Calcium binding determinations of human erythrocyte calmodulin, by equilibrium dialysis and fluorescence titration, were in close agreement with previous studies on other calmodulins.

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The objectives of the experiments presented here were to ascertain if human erythrocyte calmodulin is indeed identical with other calmodulins (9, 10) and to investigate the interaction between calmodulin and erythrocyte Ca++-ATPase.

EXPERIMENTAL PROCEDURES

Materials

Imidazole was recrystallized from benzene and then acetone. Calcium standard solutions were prepared from CaCO₃. Bovine serum albumin was from Miles Laboratories, 1,1-1-tosyalamiido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin) was from Worthington, “CaCl₂ (1 Ci/mmol) was from Amersham Radiochemical Centre, and Na₂ATP was from Sigma. Samples of bovine brain calmodulin were supplied by Dr. Jerry Wang, University of Manitoba, and Dr. Martin Watterson, Rockefeller University.

Methods

Protein was determined by the Coomassie dye binding assay (21), by the Folin method (22), by amino acid analysis, or N₂ (2.3 for erythrocyte calmodulin). Bovine serum albumin, whose concentration was determined by amino acid analysis, was the standard for the first

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3 The abbreviations used are: Ca²⁺-ATPase, calcium-activated adenosine triphosphatase; TPCK-trypsin, trypsin treated with 1,1-tosyalamiido-2-phenylethyl chloromethyl ketone; EGTA, ethylene glycol bis(β-aminocarbonyl ether)N,N,N',N'-tetraacetic acid.
two methods. The various methods of protein determination were always in close agreement.

**Purification of Erythrocyte Calmodulin**—Human erythrocyte calmodulin was prepared as previously described (10) with the following modifications. DEAE-Sephadex A-25 (3.5 mg/ml) was used throughout in place of DEAE-cellulose. In the batch steps, the benzylate was brought to 0.1 M NaCl, adjusted to pH 5.5 with 1 M acetic acid, and then 1 g of DEAE-Sephadex was added per liter. The batch-purified material was eluted (10), desalted, and lyophilized. The lyophilized protein was suspended in a minimum volume of 10 mM imidazolium chloride, pH 7.0, and mixed with an equal volume of a saturated solution of ammonium sulfate in 0.1 M imidazolium chloride, pH 7.0. The solution was clarified by centrifugation and the protein was precipitated by titration to pH 4.0 (10). The protein was dissolved in 1 to 5 ml of 50 mM NH₄HCO₃, lyophilized, and stored as the lyophilized powder at −70°C until needed. This modified procedure requires fewer manipulations and results in somewhat improved and more reproducible yields of homogeneous protein.

**Erythrocyte Membrane Preparation**—Human erythrocyte membranes were isolated as previously described (10). After the final wash in 10 mM imidazolium chloride, pH 7.0, they were diluted with an equimolar mixture of sodium and potassium chloride to a total concentration of 50 mM imidazolium chloride, pH 7.0, and centrifuged at 100,000 × g for 30 min. The pellet was resuspended in 5 volumes of 0.25 M sucrose, 1 mM mercaptoethanol, 0.5 mM MgCl₂, and 40 mM imidazolium chloride, pH 7.0, and again centrifuged. This final pellet was then rapidly frozen and stored at −70°C until needed. Ca²⁺-ATPase and the response of this activity to calmodulin remained constant for at least 6 months. In contrast, erythrocyte membranes which have not been subjected to this washing procedure lose most of their Ca²⁺-ATPase activity upon freezing. When not frozen, they lose this activity slowly over several weeks at 0°C. All of the experiments presented here were performed with a single membrane preparation for consistency and repeated on other preparations to insure reproducibility.

**ATPase Assays**—The ATPase activity of ghosts was assayed at 37°C in a medium containing 100 mM NaCl, 20 mM KCl, 0.5 mM EGTA, 0.1 mM ouabain, 3 mM MgCl₂, 3 mM Na₂ATP, and 40 mM imidazolium chloride, pH 7.0. The Ca²⁺-stimulated ATPase activity was determined by subtracting this unstimulated ATPase activity from the ATPase activity in the same medium supplemented with CalCl₂. The total CaCl₂ concentration necessary to achieve a desired free CaCl₂ concentration was calculated using an iterative computer program that takes into account binding of Ca²⁺ and Mg²⁺ to both ATP and EGTA (23, 24). Under otherwise stated, the buffered, free Ca²⁺ concentration was 30 μM, which is that required for maximal activity of the Ca²⁺-ATPase complex.

Under all circumstances, less than 20% of the ATP was hydrolyzed during the assay and ATP hydrolysis was linear with time. Calmodulin always was used to equilibrate with the enzyme on ice for 15 min before the assay was initiated with ATP. Sodium dodecyl sulfate (1.17% final concentration) was used to quench the ATPase reaction and inorganic phosphate was determined (10).

**Tryptic Peptide Mapping**—Bovine brain and human erythrocyte calmodulins (2 mg/ml) were dialyzed exhaustively against 50 mM NH₄HCO₃, 1 mM EGTA prior to digestion. TPCK-trypsin (1 mg/ml in 50 mM NH₄HCO₃, 1 mM EGTA) was added and digestion was continued for 4 h more at 37°C and the digest was lyophilized. The lyophilized digests were dissolved, spotted on a Brinkmann 300MN cellulose thin-layer plate (20 × 20 cm) (layer thickness = 0.1 mm), and submitted to electrophoresis at 500 V for 2 h. The cathode, anode, and plate buffers were all 0.02 M pyridine adjusted to pH 4.6 with acetic acid. Ascending chromatography in 1-butanol/pyridine/acetic acid: H₂O (120:30:24:96) was used. The second dimension and cadmium-ninhydrin (25) was used to stain the peptide.

**Equilibrium Dialysis**—An apparatus which allows the repeated use of the same sample of calmodulin was constructed as follows. Two pieces of dialysis tubing were mounted in a rubber stopper in such a way that one end of each was sealed and the other end was penetrated by a piece of plastic tubing. The rubber stopper was fitted to a polycarbonate Erlenmeyer flask containing the dialysis buffer. Dialyzing samples could be removed or replaced through the fine tubing.

The experiment was performed at 4°C and 37°C. Since the equilibrium constants for EGTA apply only at 20–25°C (26), Ca²⁺/EGTA buffers were not used. In the absence of Ca²⁺ buffers, acid-washed plastic laboratory ware was used throughout. At the beginning of the experiment, one of the dialysis bags in each apparatus contained 2 ml of 1 mg/ml of erythrocyte calmodulin; the other bag contained 2 ml of distilled water. Aliquots were withdrawn from each bag at appropriate times and the solution in the dialysis bag was removed and the EGTA concentration was determined in the following way. The sample was made 0.1 M NaOH and titrated with a standard CaCl₂ solution in the presence of 2-hydroxy-1-(2-hydroxy-4-sulfo-l-naphthylazo)-3-naphthoic acid (20). The EGTA concentration determined was ≤10 μM. This demonstrated that the dialysis had effectively removed the EGTA. After the third change of buffer, a sample of the inside and outside solution from both apparatuses was removed for Ca²⁺ determination by atomic absorption spectroscopy. The Ca²⁺ concentration on both sides of the dialysis membrane was less than the limit of detection (0.1 μM) of the instrument used (Instrumentation Laboratory Inc., model No. 451, flame type). Equilibrium dialysis was then initiated by adding 100 μCi of ⁴⁰CaCl₂ to the inside of the dialysis apparatus, and this sample was transferred to the other. The time course of Ca²⁺ equilibration with the solution containing calmodulin was determined; 2 days were required to reach equilibrium. The approach to equilibrium displayed the kinetics of a reversible process. After equilibrium was reached, the concentrations of Ca²⁺ and protein were determined. Since ⁴⁰Ca solutions could not be used in the atomic absorption spectrometer, the Ca²⁺ concentration present in the outside solution of the apparatus containing only CaCl₂ was determined by atomic absorption. Then, aliquots of the outside solution from both apparatuses were compared to each other and to standard CaCl₂ solutions by spectrophotometric titration (30) using standard disodium ethylenedinitrilo-tetraacetic acid solutions. The results of this titration method were always in close agreement with the atomic absorption method. For the Ca²⁺ concentration of the outside solution was used to calculate the specific activity of the Ca²⁺ and this value was used to calculate the amount bound to calmodulin. A sample of calmodulin from the nonradioactive apparatus was also analyzed for Ca²⁺ by atomic absorption and this value was always agreed well with that determined for the radioactive sample. To minimize matrix effects, all CaCl₂ standard solutions used were made up in the same buffer and salt solution used for equilibrium dialysis. Then, an appropriate volume (less than 1 ml in all cases) of a standardized, nonradioactive Ca²⁺ solution was added to the outside solution to change both the concentration and specific activity of the ligand and the experiment was repeated following closely the approach to the new concentration. At the completion of the experiment (2 to 3 weeks), the calmodulin used was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by its ability to activate the Ca²⁺-ATPase, and was found to be unaltered.

**Fluorescence Titration**—Fluorescence measurements were made at 25°C using an Aminco-Bowman (American Instruments) spectrofluorimeter equipped with a temperature-controlled water circulator and a jacketed cell holder. Erythrocyte calmodulin (1 mg/ml) was dialyzed against 100 mM NaCl, 10 mM NaNa, 50 mM imidazolium chloride, and 1.0 mM EGTA, pH 7.0, at 25°C. The protein concentration after dialysis was then lowered to 0.2 mg/ml by dilution with the same buffer. Excitation was at 290 nm and emission was measured at 320 nm. Excitation and emission slits with a band pass of 5.5 nm were used. The calmodulin solution (1.5 ml, 0.2 mg/ml) was titrated with 1- to 2-μl aliquots of a 25.0 mM CaCl₂ standard. The pH was measured after each addition. The pH changed 0.13 units (pH 7.0 to pH 6.87) during the course of the experiment. The free Ca²⁺ concentration after each solution was calculated using a computer program that also accounted for pH and dilution on the Ca²⁺-buffering capacity of EGTA. Fluorescence intensity was also corrected for dilution.

**Mathematical Methods**—All programs were written in PL/I and run on a Burroughs’ 6700 series computer. The equilibrium dialysis data was fitted to the equation

$$
\frac{\delta}{K} = \sum n_i [Ca^{2+}]
$$

where \(\delta\) refers to the moles of Ca²⁺ bound/mole of protein at each
it follows that

\[ \Delta v_i = \frac{k_{cat} [E]_{TOT} [A]}{K_A + [A]} - \frac{\Delta V_{max} [A]}{K_{app} + [A]} \]  

(5)

where

\[ k_{cat} = \frac{k_A}{1 + K_c/[Ca^{2+}]} - \frac{k_r}{1 + K_r/[Ca^{2+}]} \]

(6)

It can also be shown that

\[ \Delta v_i = k_{cat}([E]_{TOT} - [E + Ca^{2+}]) \]  

(7)

From this relationship, it can be seen that \( \Delta v_i \) is always directly proportional to the total concentration of all forms of the enzyme to which calmodulin is bound and that when \( \Delta v_i = (\Delta V_{max}/2) \),

\[ [E + A]_{TOT} = \frac{[E]_{TOT} - [E + A]_{TOT}}{[E - A]_{TOT}} \]

(8)

Finally, it is clear that

\[ K_{app} = \frac{([E]_{TOT} - [E - A]_{TOT})([A]_{TOT} - [E - A]_{TOT})}{[E - A]_{TOT}} \]

(9)

where \([E]_{TOT}\) is total concentration of calmodulin; and it follows that when \( \Delta v_i = (\Delta V_{max}/2) \),

\[ [A]_{TOT} = K_{app} + \frac{[E]_{TOT}}{2} \]

(10)

to arrive at first estimates of \([A]_{TOT}\) for each point.

Improved estimates of \([E]_{TOT}\), \(K_{app}\), and \([A]_{TOT}\) can be obtained by an iterative procedure. The current estimates of \([A]_{TOT}\) and \(\Delta v_i\) are used, in conjunction with Equation 5, to obtain a new estimate for \([E]_{TOT}\) for each erythrocyte membranes by interpolation. The interpolation is performed by weighted least squares analysis of the data directly (nonlinear least squares) or transformed into Hanes form ([A]/\(\Delta v_i\) versus \([A]\), linear least squares). The final values are unaffected by the choice of fitting procedure. Any dependence of the new \([A]_{TOT}\) on [erythrocyte membranes] is used to determine a new \(K_{app}\) by equation 9. This value and Equation 10 are used to obtain new estimates of \(K_{app}\) and \([E]_{TOT}\). These are substituted into the following equation

\[ [A] = ([E]_{TOT} - [E - A]_{TOT})/[E - A]_{TOT} + 4 [A]_{TOT} K_{app} \]  

(11)

and \([A]_{TOT}\) is concentration of free calmodulin, \([E]_{TOT}\) is concentration of free \(Ca^{2+}\)-ATPase, \([E - \cdot \cdot \cdot Ca^{2+}]_{TOT}\) is concentration of occupied \(Ca^{2+}\) sites associated with empty calmodulin sites, \([A - \cdot \cdot \cdot Ca^{2+}]_{TOT}\) is concentration of occupied \(Ca^{2+}\) sites associated with occupied calmodulin sites, and \(P\) refers to the products of the ATPase reaction. It has been shown (28) that the expression for the initial velocity of an enzymatic reaction under these circumstances is

\[ v_i = \frac{k_1 + k_2 K_c/[Ca^{2+}][E]_{TOT}}{1 + K_c/[Ca^{2+}] + K_r/[Ca^{2+}]} \]  

(1)

where

\[ [E]_{TOT} = [A \cdot E] + [E \cdot Ca^{2+}] + [A \cdot \cdot \cdot Ca^{2+}] \]

(2)

and is equal to the total concentration of sites. The difference between the initial velocity in the presence of calmodulin and that in the absence of calmodulin, \(\Delta v_i\), at any \([Ca^{2+}\]) is

\[ \Delta v_i = \frac{k_{cat} (1 + K_c/[Ca^{2+}])}{k_r (1 + K_r/[Ca^{2+}])} [E]_{TOT} [A] \]

and 

\[ K_{app} = \frac{[E]_{TOT} + [E + Ca^{2+}]_{TOT}}{[A \cdot E] + [A - \cdot \cdot \cdot Ca^{2+}]} \frac{k_1 (1 + K_c/[Ca^{2+}])}{1 + K_r/[Ca^{2+}]} \]

(4)

There are several advantages to this formulation. By dealing only with \(\Delta v_i\), all \(Ca^{2+}\)-ATPase activities which are not directly affected by calmodulin are automatically eliminated. Furthermore, the function is a rectangular hyperbola in \([A]\). Finally, there is a direct relationship between this equation and the apparent dissociation constant for the interaction between enzyme and calmodulin. Since

\[ \Delta v_i = \frac{k_{cat} (1 + K_c/[Ca^{2+}])}{k_r (1 + K_r/[Ca^{2+}])} [E]_{TOT} [A] \]

(3)

Preparation of Membranes Stripped of Spectrum—Spectrin and actin were removed from erythrocyte membranes by a modification of an established procedure (29). The membranes were centrifuged at 40,000 \(\times g_{max}\), and the supernatant was carefully removed. The pellet was resuspended in 172 mM (iso-osmotic) Tris-HCl, pH 7.6, and left on ice for 30 min. The membranes were then pelleted at 100,000 \(\times g_{max}\), and the spectrin-actin was extracted by diluting the pellet 25-fold with 0.1 mM NaATP, 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol, pH 8.0, and dialyzed versus 100 volumes of the same solution overnight. The membranes were then pelleted at 100,000 \(\times g_{max}\), and the spectrin-actin was extracted by diluting the pellet 25-fold with 0.1 mM NaATP, 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol, pH 8.0, and dialyzed against 0.1 M sucrose, 0.5 mM MgCl₂, 1 mM 2-mercaptoethanol, and 40 mM imidazole chloride, pH 7.0, and stored on ice until needed. The supernatants were concentrated
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5-fold by vacuum dialysis and also stored on ice. Sodium dodecyl sulfate-acrylamide gel electrophoresis of the various fractions and the starting material demonstrated that the supernatants contained the majority of the spectrin and actin present initially. This fraction also contained detectable amounts of Bands 4 and 6 (30). The extracted membranes were for the most part devoid of these bands. The relative amounts of the various proteins present in the starting material and the final membrane preparation were determined by integration of the Coomassie staining intensity of gels scanned for absorbance at 550 nm. It was assumed that Band 3 (30) remained membrane-bound during the extraction procedure and therefore could be used as a measure of membrane concentration.

Total lipid phosphorus of all fractions was determined after oxidative degradation with Mg(NO₃)₂ (31).

RESULTS

Peptide Maps—In Fig. 1, tryptic peptide maps of human erythrocyte calmodulin, bovine brain calmodulin, and a mixture of these two proteins are presented. It is clear from examination of this figure that there are more peptides on each map than would be expected from the lysine and arginine content of calmodulin (lysine + arginine = 13 residues/molecule). Stevens et al. (32), however, also reported 27 to 28 peptides in their tryptic peptide maps of bovine heart and bovine brain calmodulins. The reason for this large number of peptides is unclear. Digestion under more strenuous conditions (trypsin/calmodulin, 1:33 at 37°C for 24 h) than those used routinely gave the same number of peptides.

All 29 major and minor peptides on the erythrocyte map are also present both on the bovine map and the map of a 1:1 mixture of the two digests. All of the spots on the mixture map also occur on the bovine map. As the relative amount of bovine calmodulin in the mixture is decreased, the map of the mixture becomes more like that obtained with the erythrocyte digest alone (data not shown). These observations demonstrate that each tryptic peptide in the digest of the erythrocyte protein corresponds to a peptide which is present in the bovine digest and which is indistinguishable from it by the peptide mapping procedure. Therefore, the bovine protein contains a discrete set of tryptic peptides which are derived exclusively from a length of sequence which is directly homologous to the complete erythrocyte sequence.

It is also apparent (Fig. 1) that the bovine digest contains 5 to 7 peptides not found in the erythrocyte digest. This may be connected with the effects of storage on calmodulin. While calmodulin freshly isolated from brain, testis, or erythrocytes is homogeneous on sodium dodecyl sulfate-gel electrophoresis, upon storage of all three proteins a second component begins to appear (10). For the peptide mapping studies presented here, the samples of bovine brain calmodulin contained detectable amounts of this second component (about 50% of one sample and 10% of the other), while the erythrocyte calmodulin was homogeneous. For the experiment presented (Fig. 1), erythrocyte calmodulin was compared to the sample of the bovine brain protein which had the smallest amount of this second component. To determine whether the 5 to 7 extra peptides on the bovine map might be associated with this second electrophoretic component, the two samples of bovine calmodulin which contained different amounts of the second component were digested and their maps were compared. Both digests gave peptide maps with the same number of spots and the same pattern, but the 5 to 7 peptides unique to
the bovine map were relatively more intense in the map of that sample which contained the greatest amount of the second electrophoretic component. Since it was not possible to separate the two components of the bovine sample preparatively, it cannot be definitely shown that these unique peptides are derived from the second component, though this seems likely.

**Equilibrium Dialysis**—Equilibrium dialysis measurements of Ca\(^{2+}\)-binding to erythrocyte calmodulin at 4°C and 37°C are presented in Fig. 2. The curve drawn is the analytical fit of the 4°C data. The data obtained at 37°C are included on the same figure for comparison. The data were fitted by an iterative least squares procedure to the equation

\[ t = \frac{\sum_{i=1}^{i} n_i [Ca^{2+}]}{\sum_{i=1}^{i} K_i + [Ca^{2+}]} \]

The program allows \( i \) to be set at any integral value. For the curve drawn (\( i = 2; 4^\circ \)C data), \( n_1 = 3.5, K_1 = 7.6 \mu M; n_2 = 5.3, K_2 = 42.4 \mu M \). If, however, \( i \) is allowed to increase, it becomes apparent that there is one set of sites (\( n_1 = 3.8 \pm 0.3, K_1 = 7 \mu M \)) that are unique and several other kinds of sites which are poorly defined and which are only significant contributors at higher [Ca\(^{2+}\)]. This follows from the observations that as \( i \) is allowed to increase, \( n_i \) and \( K_i \) do not change, the sum of the squares decreases indicating a better fit, and the additional \( n \) and \( K \) pairs all have \( K > 15 \mu M \). These considerations indicate that it is the data at higher [Ca\(^{2+}\)] that is fit better by a more complex equation (\( i > 2 \)).

As the equation becomes more complex, the uncertainty in the constants becomes great. Only those constants for \( i = 2 \) are presented realizing that the \( n_i \) and \( K_i \) represent a group of sites filled only at high [Ca\(^{2+}\)]. It can be concluded that there are four specific calcium sites on the protein but that at higher [Ca\(^{2+}\)], calcium can also be bound at anionic sites elsewhere on the surface of this highly acidic protein. Such sites would be expected to be a heterogenous mixture.

**Fluorescence Changes upon Ca\(^{2+}\)-binding**—Fig. 3 presents the effect of [Ca\(^{2+}\)] on the fluorescence of erythrocyte calmodulin at 25°C. A 2-fold enhancement of the fluorescence was observed in the presence of saturating amounts of Ca\(^{2+}\). The apparent dissociation constant is 0.92 \( \mu M \) in agreement with Dedman et al. (33) who found 0.85 \( \mu M \) by the same technique for rat testis calmodulin (determined from the data presented in Fig. 4 of Ref. 33). These values are the same within experimental error and provide further evidence of the identity of erythrocyte calmodulin and calmodulin from another source.

**Determination of Ca\(^{2+}\)-stimulated ATPase Concentration**—Fig. 4 presents an experiment designed to measure the number of sites to which calmodulin binds on the erythrocyte membrane. Under the experimental conditions used (30 \( \mu M \) [Ca\(^{2+}\)]\(_{free}\)), saturating amounts of calmodulin activate the Ca\(^{2+}\)-ATPase 3.8-fold and unstimulated Ca\(^{2+}\)-ATPase activity has been subtracted from the data. The data shown in Fig. 4A are completely consistent with what would be expected if the concentrations of sites present under the various assay conditions were similar in magnitude to \( K_{app} \). By visual interpolation, [A]\(_{0.5} \) was determined for each membrane concentration and these data are presented in Fig. 4B. Using weighted nonlinear regression analysis rather than visual interpolation to obtain estimates of [A]\(_{0.5} \) gave the same values ± 5%. From Fig. 4B, [E\(_{TOT}\) (17.1 pmol.mg protein\(^{-1}\)) and \( K_{app} \) (15.6 nM) were calculated. Refinement of these estimates by an iterative procedure gave [E\(_{TOT}\) = 16.5 pmol.mg\(^{-1}\) and \( K_{app} \) = 14.5 nM. The free concentration of calmodulin for each point was calculated with these parameters and the data was replotted in terms of these values (Fig. 4C). As shown in Fig. 4C, when 1/\( \Delta n \) is plotted against 1/[A]\(_{free}\) instead of 1/[A]\(_{TOT}\), the data for all membrane concentrations superimpose upon a single straight line as expected. The \( K_{app} \) (Fig. 4D) and \( V_{max} \) (Fig. 4C) determined from such plots are independent of membrane concentration (\( V_{max} = 40.6 \pm 1.3 \) nmol.min\(^{-1}\)mg\(^{-1}\)). In Fig. 4E, an Eadie plot of the corrected data is also included.

Although kinetic data can only be compatible with a proposed model and cannot prove it, the following experimental observations argue that the kinetic mechanism proposed, or some portion of it, is the one which actually governs the situation. The plots of 1/\( \Delta n \) against 1/[A]\(_{free}\) (Fig. 4A) show precisely those characteristics predicted by the kinetic mech-

![Fig. 2. Equilibrium dialysis measurements of Ca\(^{2+}\)-binding to erythrocyte calmodulin. The experiments presented were performed at 4°C (○) and 37°C (△). The curve shown is that fitted to the data at 4°C. The 37°C data is presented on the same figure for comparison.](image)

![Fig. 3. The effect of Ca\(^{2+}\)-binding on the fluorescence emission of erythrocyte calmodulin. Excitation was at 280 nm and emission at 320 nm. The maximum change represents a 2-fold enhancement of this fluorescence. Ca\(^{2+}\)/EGTA buffers were used and the Ca\(^{2+}\) concentrations are calculated free concentrations. Human erythrocyte calmodulin was 0.2 mg/ml and the temperature was 25°C.](image)
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FIG. 4. Kinetic titration of calmodulin binding sites. A to E, kinetic data of a single set of experiments arranged to show how \([E]_{\text{TOT}}\) and \(K_{\text{app}}\) are calculated. A, double reciprocal plots of the calmodulin-activated Ca\(^{2+}\)-ATPase activity \((\Delta v)\) against the total concentration of human erythrocyte calmodulin added \([A]_{\text{TOT}}\). B, the concentration of calmodulin required for 50% of maximal stimulation \([A]_{0.5}\), determined by visual interpolation, is plotted against erythrocyte membrane concentration. The intercept is equal to \(K_{\text{app}}\) at the Ca\(^{2+}\) concentration used (buffered at 30 \(\mu M\)) and the slope equals \([E]_{\text{TOT}}/2\) for unit membrane concentration. Refined estimates of these parameters were then used to calculate the actual, free concentration of calmodulin for each point in A, and these data are replotted in C. D, the calculated, free concentration of calmodulin at which 50% of maximal effect occurs \((K_{\text{app}})\) is plotted against membrane concentration for comparison with B. E, plot of \(\Delta v\) against \(\Delta v/[A]_{\text{PHER}}\). The units of \(\Delta v\) are nmol \cdot min\(^{-1}\) \cdot mg\(^{-1}\) in A and C and nmol \cdot min\(^{-1}\) in E. Concentrations of calmodulin, \([A]_{0.5}\), and \(K_{\text{app}}\) are expressed as nanomolar, and the erythrocyte membrane concentrations used (mg/ml, final concentration) were: 2.28 (○), 1.90 (△), 1.33 (□), 0.95 (■), 0.57 (△), and 0.19 (×). In A, the data at higher concentrations of calmodulin have only been included for the highest (○) and lowest (×) membrane concentrations used to simplify the figure; the data for all other membrane concentrations fall in between these extremes. In D, all of the data at high concentrations of calmodulin could not be included on one figure, so a random sample is presented.

Calmodulin activates Ca\(^{2+}\)-ATPase by binding directly to the enzyme at a specific site in a simple, reversible association.

Association of Calmodulin-activated Ca\(^{2+}\)-ATPase with the Plasma Membrane—There have been several reports of a Ca\(^{2+}\)-ATPase activity associated with isolated spectrin and actin. A discussion of this problem has been presented, and experimental results which suggest that this ATPase might result from membrane contamination of the isolated spectrin fractions have been described (29). Regardless of the outcome of this controversy, if a Ca\(^{2+}\)-ATPase associated with spectrin interacts with calmodulin, this would complicate the kinetic results described above. To clarify this issue, the following experiment was performed. Spectrin and actin were separated from the erythrocyte membrane and the ATPase activity, total lipid phosphate, and protein species present in these two fractions were determined. The results are shown in Table I. It is clear from these data that the ATPase activity is not associated with either spectrin or actin but is associated with the erythrocyte membrane. In addition, the total lipid phosphate and protein species present in these two fractions are not associated with the ATPase activity. Therefore, the ATPase activity observed in these fractions is not due to spectrin or actin contamination.

From these observations, it is concluded that calmodulin activates Ca\(^{2+}\)-ATPase by binding directly to the enzyme at a specific site in a simple, reversible association.
fractions were determined. The smooth membrane fraction contained 49% of the total Ca\textsuperscript{2+}-ATPase activity of the starting material and 88% of the lipid phosphate. Negligible amounts of Mg\textsuperscript{2+}-ATPase, Ca\textsuperscript{2+}-ATPase (<1 nmol-min\textsuperscript{-1}·mg\textsuperscript{-1}), and lipid phosphate were detected in the spectrin-actin fraction. Furthermore, no additional Ca\textsuperscript{2+}-ATPase activity was detected when calmodulin (final concentration = 300 nM) was added to the assay. In contrast, the Ca\textsuperscript{2+}-ATPase activity of the extracted membranes, which retained less than 5% of the spectrin and actin present in the starting material, was activated by calmodulin by the same factor as that measured for the starting material. The extraction procedure used removed 41% of the protein from the membrane.

**Discussion**

Erythrocyte calmodulin has been shown to be indistinguishable from calmodulins from other sources (10). The proportion examined previously include apparent molecular weight, isoelectric point, amino acid composition, and biological activity. Another indication of this close similarity are the peptide maps presented in Fig. 1. The fact that the equivalents of all 29 peptides of erythrocyte calmodulin are present in bovine calmodulin leads to the conclusion that within the limits of peptide mapping to detect subtle differences, bovine brain and human erythrocyte calmodulins are very similar, if not identical, proteins. A consideration of results obtained with various muscle and cytoplasmic actins will serve to put the present observations in perspective. Although cytoplasmic and muscle actins are indistinguishable by at least one peptide mapping procedure (34), it has been demonstrated that they differ in sequence at as many as 5% of the positions (35). It should be noted, however, that these replacements are highly conservative. It is our conclusion that the similarity between the sequences of the bovine and human calmodulins is strong and that there are, at most, only minor, highly conservative differences between them.

The additional peptides on the map of the bovine brain calmodulin trypsinic digest (Fig. 1) complicate this conclusion somewhat, however, and require consideration. The fact that the apparent molecular weights of the brain and erythrocyte calmodulins are indistinguishable (10) precludes the possibility that bovine calmodulin is more than 5 residues longer than erythrocyte calmodulin. The fact that the calmodulins have indistinguishable amino acid compositions is inconsistent with the presence of extra cleavage points for trypsin. These considerations and others presented with the results suggest that either the 5 to 7 unique peptides on the bovine map are due to an impurity, to the heterogeneity observed on storage, or to a partial modification of some of the 29 peptides which are equivalent during the purification procedure.

The equilibrium dialysis measurements (Fig. 2) and the fluorescence titration (Fig. 3) demonstrate that erythrocyte calmodulin is indistinguishable from other calmodulins in its ability to bind Ca\textsuperscript{2+} as well. In the fluorescence study (Fig. 3), the [Ca\textsuperscript{2+}] required for 50% effect (0.9 μM) and the magnitude of the fluorescence enhancement (2-fold at saturating [Ca\textsuperscript{2+}]) were indistinguishable from those found for another calmodulin. The number of high affinity binding sites and the value of the dissociation constant (Fig. 2) are in reasonable agreement with those expected of a simple, noncovalent equilibrium between calmodulin and Ca\textsuperscript{2+}. Since it has been established that calmodulin influences several general control processes within the cell, it was possible that its effect on Ca\textsuperscript{2+}-ATPase from the erythrocyte was mediated in some indirect fashion. In this report, however, it has been demonstrated that the activation occurs in membranes stripped of spectrin and actin and in the absence of any cytoplasmic cofactors with the exception of Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-ATP. Furthermore, the kinetic features of the interaction between calmodulin and Ca\textsuperscript{2+}-ATPase conform closely to those expected of a simple, noncovalent equilibrium between a ligand and a defined quantity of enzyme (Fig. 4). These observations conclusively rule out any complicated chain of events between calmodulin and the actual activation of this enzyme.

An additional advantage of this experiment is that a measurement of the concentration of enzyme-linked sites that bind calmodulin can be made. This has been done by a simple kinetic approach which allows such a determination on an impure enzyme. The value of \([E]_\text{TOT}\) derived from these data corresponds to 4500 sites-erythrocyte \textsuperscript{-1} (assuming 5 × 10\textsuperscript{-10} mg of membrane protein-erythrocyte \textsuperscript{-1} (29)\textsuperscript{2} and a turnover number for the activated form of the enzyme of about 3000 μmol of P\textsubscript{i}·μmol of site\textsuperscript{-1}·min\textsuperscript{-1}. The Ca\textsuperscript{2+}-ATPase which is activated by calmodulin is known to catalyze the active transport of Ca\textsuperscript{2+} (20). This value for the turnover number agrees satisfactorily with turnover numbers calculated for other membrane-bound, transport ATPases. The turnover number for the Ca\textsuperscript{2+}-ATPase from sarcoplasmic reticulum is 3800 μmol of P\textsubscript{i}·μmol of chain\textsuperscript{-1}·min\textsuperscript{-1} (calculated from the data in Table 1 of Ref. 37, using a chain molecular weight of 120,000) and that for (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase from renal medulla is 6000 μmol of P\textsubscript{i}·μmol of chain\textsuperscript{-1}·min\textsuperscript{-1} (38, 39).

The function of calmodulin in the erythrocyte appears to be the control of the Ca\textsuperscript{2+} transport ATPase which maintains the cytosol [Ca\textsuperscript{2+}] at micromolar levels. Since the cytoplasmic [Ca\textsuperscript{2+}] influences the deformability of the cell (40, 41) and its survival time in circulation, the control of this Ca\textsuperscript{2+}-ATPase is important to the overall circulatory physiology of the organism. Since the concentration of calmodulin in the cytoplasm is greater than 1 μM (calculated from the data in Table 1 of Ref. 10) and the apparent dissociation constant and concentration of enzyme is certainly less than this value (Fig. 2). It should be noted that the agreement of the results in Fig. 4 with the mathematical expectations are consistent with the conclusion that Ca\textsuperscript{2+}-ATPase is the only protein in the erythrocyte membrane which binds calmodulin at this level of occupancy and with high affinity.
4), there is sufficient calmodulin in the cell to exert its full effect on calcium transport.

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