Photochemical Cross-linking of Azidocalmodulin to the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of the Erythrocyte Membrane*

(Received for publication, February 23, 1981, and in revised form, April 27, 1981)

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The calmodulin (CaM)-binding proteins present in the human red blood cell membrane were examined through photoaffinity labeling. Two different membrane preparations were used: white ghosts and inside-out vesicles. These were incubated with azido-\(^{125}\)I-CaM, a photoactivatable derivative of calmodulin, and photoactivated. Cross-linked products were identified by autoradiography of dried sodium dodecyl sulfate slab gels and quantitated by scanning gel slices for \(^{125}\)I. The major product formed with each membrane type had an apparent \(M_c\) of 168,000. No other product was common to both membrane types; however, a few other products unique to each vesicle type were detected. The formation of the 168,000-dalton product correlated with an increased basal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity in white ghosts and with an increased basal Ca\(^{2+}\) transport in inside-out vesicles. This suggests that the 168,000-dalton product represents a cross-link between azido-\(^{125}\)I-CaM and the Ca\(^{2+}\)-pump ATPase. The ATPase appears to be a single protein of about 150,000 daltons, which can bind a single calmodulin. No evidence was found to indicate anything other than a 1:1 binding stoichiometry between calmodulin and the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase.

Isolated plasma membranes (white ghosts) from the human erythrocyte contain a Ca\(^{2+}\)-dependent, Mg\(^{2+}\)-dependent ATPase that responds to stimulation by calmodulin (1, 2). Inside-out vesicles prepared from erythrocytes can accumulate Ca\(^{2+}\) against an electrochemical gradient. This active transport of Ca\(^{2+}\) is also stimulated by calmodulin (3, 4). The existing evidence suggests that these two enzyme systems are one and the same; the Ca\(^{2+}\)-pump ATPase, which is thought to be responsible for the maintenance of low intracellular Ca\(^{2+}\) concentration (5–7).

The catalytic properties of the ATPase have been suggested to reside in a single polyepitope of apparent \(M_c\) = 140,000–150,000 (8–10). Detergent solubilization and gel filtration techniques allowed Wolf et al. (11) to obtain a highly enriched (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase fraction. This preparation contained distinct protein subunits, with apparent \(M_c\) of 145,000, 115,000, and 105,000. The 145,000-dalton subunit could be phosphorylated in the presence of \([\gamma^3P]ATP\). Affinity chromatography of a similar preparation over calmodulin-Sepharose resulted in one band detectable by SDS-gel electrophoresis (12).

* This work was supported in part by Grants HL 23806 and AM 16436 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of National Institutes of Health Postdoctoral Fellowship GM 07758.

The abbreviations used are: SDS, sodium dodecyl sulfate; RBC, red blood cell; \(I_w\), white ghosts prepared from hypotonic hemolyzed.

Recentl, a photoactivatable, radioactive derivative of calmodulin (azido-\(^{125}\)I-CaM) has been synthesized and shown to be effective in specifically cross-linking with calmodulin binding proteins in a Ca\(^{2+}\)-dependent manner (27). In the present paper, we report on the use of the photoactivatable calmodulin to label two different membrane preparations from erythrocytes. A 150,000-dalton protein becomes labeled in a concentration-dependent manner, and the amount of product formed correlates with increased basal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity and increased basal Ca\(^{2+}\) transport. Additional proteins can cross-link with azido-\(^{125}\)I-CaM in a Ca\(^{2+}\)-dependent manner. The appearance of these latter products is dependent upon the type of membrane preparation used.

MATERIALS AND METHODS

Radioisotopes were purchased from New England Nuclear. Bovine serum albumin, \(\beta\)-galactosidase, ovalbumin, and aldolase, used as molecular weight markers in SDS gels, were obtained from Sigma. An additional marker, rabbit skeletal myosin, was graciously provided by Dr. Arthur M. Edelman. Methyl-4-azidobenzoimidate was purchased from Pierce. All other reagents were the best grade available from commercial sources.

Preparation of I\(_o\) Membranes—Outdated packed human RBCs were obtained from the local blood bank. The cells were washed four times in ice-cold isotonic saline (0.9% NaCl), and the supernatant anduffy coat were removed by aspiration. Hemolysis of the RBCs in 26% ideal mannitol imidazole buffer (pH 7.4) and subsequent isolation of the white membrane ghosts were done according to the procedure described by Farrance and Vincenti (28).

Preparation of Inside-Out Vesicles—Fresh blood was obtained in 20 ideal mannitol imidazole, pH 7.1; IOV, inside-out vesicles; CaM, calmodulin; EGTA, ethylene glycol bis\(\beta\)-aminoethyl ether) \(N,N,N',N'\)-tetraacetic acid.
from healthy adult volunteers. The blood was centrifuged at 2,000 rpm for 5 min (Sorvall SS-34 rotor) prior to removal of plasma anduffy coat by aspiration. The remaining cells were washed four times with ice-cold isotonic saline. IOV were prepared and purified as previously described (29). The percentage of IOV was determined by the latency of acetylcholinesterase activity in the presence and absence of Triton X-100 (30). Typically, the IOV membranes were 72-74% inside-out and occasionally as high as 85%. The remaining membranes were "unsealed," as determined by the accessibility of glyceraldehyde-3-phosphate dehydrogenase (30).

Calcium transport experiments and ATPase assays were performed as previously described (29, 31). Due to the amount of IOV membranes used the total volume of the ATPase assay incubation solution was reduced to 0.5 ml and the reaction was stopped by the addition of 0.25 ml of 10% SDS.

Calmodulin was isolated from human RBCs (32) or bovine brain (33), and iodinated by the method of LaPorte and Storm (34). The specificity activity of $^{125}$I-Cal was $8 \times 10^4$ cpm/nmol. Azido-Cal and azido-$^{125}$I-Cal were prepared from methyl-4-azidobenzoimidate as described by Andreasen et al. (27). The product contained one azido group/Cal. Azido-Cal and membranes were incubated in the dark at room temperature in a total volume of 150 $\mu$l containing membranes (1-3 mg of protein/ml), azido-Cal (1-2 $\mu$g/ml as specified), unmodified Cal (150 $\mu$g/ml when present), 0.1 mM Mg$^{2+}$, 0.1 mM Ca$^{2+}$ (when present), 0.2 mM EGTA (when present) in 25 mM phosphate, pH 7.1. After 15 min, the incubation mixture was diluted with an equal volume of ice-cold buffer (containing either Ca$^{2+}$ or EGTA) and spun for 5 min in an Eppendorf Model 5412 microcentrifuge. Membranes were resuspended with 300 $\mu$l of buffer and recentrifuged. The final pellet was resuspended in 150 $\mu$l of buffer and irradiated for the indicated times on ice using a Mineralight UVS-11 lamp positioned 1 cm above the sample. The photolyzed product was mixed with an equal volume of SDS sample buffer, heated at 90 °C for 4 min, and electrophoresed according to the procedure of Laemmli (35). Stained gels were dried and autoradiographed at -80 °C using a DuPont Cronex Lightning Plus (YG) intensifying screen and Kodak XR-5 x-ray film. Dried gels were sliced and counted for $^{125}$I in a gamma counter.

Protein concentrations were estimated by the procedure of Peterson (36). IOV-RBC membrane equivalent was estimated by relating the acetylcholinesterase activity/mg of IOV protein to the acetylcholinesterase activity/RBC (30). RBCs were counted in a Coulter Counter Model ZBI.

RESULTS

When isolated membranes were mixed with azido-$^{125}$I-Cal and photolyzed, many labeled bands were revealed by autoradiography (Fig. 1b). This was observed with both IOV and IOV. Vincenzi et al. (37) have reported that when calmodulin binds to membranes in the presence of 0.1 mM Ca$^{2+}$, the complex does not dissociate while low levels of Ca$^{2+}$ are present. This suggested that the incubation solutions could be pelleted and washed prior to photolysis in order to remove unbound azido-$^{125}$I-Cal, while leaving the long lived azido-$^{125}$I-Cal-protein complexes intact. Fig. 2 shows the autoradiogram patterns obtained upon cross-linking azido-$^{125}$I-Cal to IOV and IOV under these conditions.

In the presence of Ca$^{2+}$ (Fig. 2, lanes b and c) several cross-linked products were detected. Inclusion of EGTA in the incubation mixture (Fig. 2, lanes a and d) or preincubation of the membranes with unmodified calmodulin (data not shown) prevented the formation of the cross-linked products. The major product formed with both IOV and IOV migrated with apparent molecular weight of 180,000 (Table I). No other product was common to both membrane types. Calmodulin has been shown to run on SDS-gel electrophoresis with an apparent $M_r$ of 20,000-21,000 when a Ca$^{2+}$ chelator was included in the sample buffer (27, 38). Assuming a molecular weight of 140,000-150,000 for the Ca$^{2+}$-Cal trigger, the 180,000-dalton labeled product falls within the range expected for a 1:1 cross-link between azido-$^{125}$I-Cal and the ATPase pump.

It was of interest, therefore, to look for functional changes in the ATPase pump, representing covalent and active cross-links,
which corresponded to the formation of the 168,000-dalton product.

**Time of photolysis**—The time required for maximum phototoxicated cross-linking was examined to find the minimum time necessary for photolysis (Fig. 3A). Increasing time of photolysis in the presence of 0.1 mM Ca**+** did not reveal any new autoradiographic bands not visualized at shorter times. Spots on the dried gel corresponding to the observed cross-linked products were cut out and counted for radioactive azido-CaM at concentrations ranging from 1.8 × 10**-8** to 2.0 × 10**-7** M. After washing and photolyzing the membranes, as described under "Materials and Methods," the membranes were washed two additional times, first in 0.5 mM EGTA, 40 mM histidine/imidazole buffer, pH 7.1, and then in 40 mM histidine/imidazole buffer, pH 7.1, to remove any noncovalently bound azido-CaM. Aliquots were then assayed for (Ca**+** + Mg**+**)-ATPase activity. Cross-linked samples were first assayed for activity in the absence of added CaM (Table II, basal) then in the presence of 2 × 10**-7** M added CaM (Table II, maximum activation). An elevation in basal activity was observed. The magnitude of the elevation increased with increasing azido-CaM in the cross-linking incubation (Table II). All samples were stimulated to the same maximal activity by addition of the exogenous CaM to the assay mixture.

The increase in basal activity and the lack of a decline in maximal activity suggested that azido-CaM was cross-linking to the ATPase. Furthermore, the cross-linking was occurring in such a way as to retain enzymatic stimulation, rather than inducing inhibition.

The activity response to cross-linking was repeated with azido-**125**I-CaM, at a concentration range of 9 × 10**-9** to 2 × 10**-7** M. Although the iodinated CaM derivative was not as potent as the nonradioactive azido-CaM in elevating basal activity, a 2- to 3-fold increase was observed, with no decrease in maximal CaM stimulation (Table II). An aliquot of each photolyzed product was electrophoresed and autoradiographed as described under "Materials and Methods" (Fig. 4A). The dried gel was then sliced and counted for **125**I. The elevation in basal activity correlated with an increase in formation of the 168,000-dalton product (Fig. 4B).

**Correlation of cross-linking with Increased Basal Ca**2+-**Transport**—IOV were photocross-linked with various concentrations of azido-**125**I-CaM, ranging from 6 × 10**-9** to 2 × 10**-7** M. A portion of each photolyzed sample was electrophoresed

![Fig. 3. Dependence of photocross-linked product formation on irradiation time. IOV (0.79 mg of protein/ml) were incubated with 2 × 10**-7** M azido-**125**I-CaM, washed, and photolyzed as described under "Materials and Methods." The irradiation time was varied from 0 to 12 min as indicated. A, 37.5 μl of each photolyzed sample was electrophoresed (7.5% SDS-polyacrylamide slab gel) and autoradiographed as described under "Materials and Methods." B, the dried gel was sliced and areas corresponding to autoradiographic bands were counted for **125**I. Zone A corresponds to the 168,000-dalton product. Unmodified calmodulin co-migrates with zone D.](image)

![Fig. 4. Dependence of cross-linked product formation and (Ca**+** + Mg**+**) ATPase activity on azido-**125**I-CaM concentrations in the incubation mixture: lane A, 9.0 × 10**-7** M; lane B, 1.8 × 10**-7** M; lane C, 5.1 × 10**-7** M; lane D, 8.9 × 10**-7** M; lane E, 2.0 × 10**-7** M. B, 20 μl of the irradiated product was analyzed for (Ca**+** + Mg**+**) ATPase activity, as described under "Materials and Methods." Slices of the dried gel corresponding to the 168,000-dalton cross-linked product (middle autoradiographic band) were counted for **125**I.](image)
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**Fig. 5.** Dependence of cross-linked product formation and Ca\(^{2+}\) transport on azido\(\_125\)I-CaM concentration. IOV (3.04 mg of protein/ml) were incubated with increasing concentrations of azido-\(\_125\)I-CaM for 15 min at room temperature and photolyzed as described under "Materials and Methods." A, 25 μl of the final irradiated product was electrophoresed (7.5% SDS-polyacrylamide slab gel) and autoradiographed (18-hr exposure) as described under "Materials and Methods." Samples contained the following azido-\(\_125\)I-CaM concentrations in the initial incubation mixture: lane A, 6 × 10\(^{-8}\) M; lane B, 1.2 × 10\(^{-8}\) M; lane C, 2.4 × 10\(^{-8}\) M; lane D, 4.9 × 10\(^{-8}\) M; lane E, 9.8 × 10\(^{-8}\) M; lane F, 2 × 10\(^{-7}\) M. B, 100 μl of the irradiated product was used to measure \(^{45}\)Ca\(^{2+}\) transport. Initial rates were obtained over a 3-min period. Basal and maximally activated rates (5 × 10\(^{-8}\) M calmodulin in transport assay) were determined using photolyzed IOV which had no azido-\(\_125\)I-CaM present in the incubation mixture. Slices of the dried gel corresponding to the location of the 168,000-dalton cross-linked product (top autoradiographic band) were counted for \(^{125}\)I.

**Table II**

| Pretreatment | (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase | Basal | Maximum activation | Activation |
|--------------|--------------------------------|-------|-------------------|------------|
|              | [nmol Pi/mg/min]               | fold  |                   |            |
| 1. None\(^a\) | 9.64                          | 58.8  | 6.1               |            |
| 2. None\(^b\) | 11.0                          | 55.9  | 5.1               |            |
| 3. 2.0 × 10\(^{-8}\) M azido-\(\_125\)I-CaM + EGTA\(^c\) | 8.47 | 56.6  | 6.7               |            |
| 4. 9.0 × 10\(^{-8}\) M azido-\(\_125\)I-CaM | 12.7 | 56.3  | 4.4               |            |
| 5. 1.8 × 10\(^{-8}\) M azido-\(\_125\)I-CaM | 14.0 | 57.5  | 4.1               |            |
| 6. 5.1 × 10\(^{-8}\) M azido-\(\_125\)I-CaM | 19.1 | 58.9  | 3.1               |            |
| 7. 8.9 × 10\(^{-8}\) M azido-\(\_125\)I-CaM | 24.3 | 55.1  | 2.3               |            |
| 8. 2.0 × 10\(^{-7}\) M azido-\(\_125\)I-CaM | 26.0 | 53.4  | 2.1               |            |
| 9. None\(^a\) | 7.85                          | 47.3  | 6.0               |            |
| 10. 1.8 × 10\(^{-8}\) M azido-CaM | 25.8 | 52.8  | 2.4               |            |
| 11. 5.1 × 10\(^{-8}\) M azido-CaM | 29.0 | 50.7  | 1.8               |            |
| 12. 8.9 × 10\(^{-8}\) M azido-CaM | 31.0 | 50.5  | 1.6               |            |
| 13. 2.0 × 10\(^{-7}\) M azido-CaM | 32.6 | 53.1  | 1.6               |            |

**DISCUSSION**

Azido-\(\_125\)I-CaM is a useful molecular probe for the identification and study of calmodulin binding proteins (21). This photoactivatable derivative of calmodulin has been used to study CaM-protein interactions in intact membrane, solubilized membrane, and purified protein systems. The proteins studied have been single polypeptide and multisubunit enzymes. The general utility of this probe suggested that it could be useful in further characterization of the erythrocyte (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase. This enzyme displays two dynamic functions, an ATPase activity and a Ca\(^{2+}\)-pumping activity (5-7). Both functions are stimulated by calmodulin (1-4) in a Ca\(^{2+}\)-dependent manner. Previous work has indicated that the ATPase and Ca\(^{2+}\)-pumping activities are associated with a protein of apparent M\(_s\) = 140,000-155,000 (8-14). The present study presents further evidence supporting this. In addition, we provide definitive evidence that the Ca\(^{2+}\)-dependent calmodulin-binding capacity residues in the same polypeptide. The cross-linking pattern indicates a 1:1 binding stoichiometry.

Photolysis of intact erythrocytes incubated with azido-\(\_125\)I-CaM revealed no cross-linked products detectable by autoradiography (data not shown). This is consistent with the accepted role of calmodulin as a regulator of intracellular proteins (40). Cross-linking studies were then carried out with two different types of erythrocyte membrane preparations which expose intracellular membrane proteins to the external medium. The I\(_{50}\) preparation is composed of "leaky" ghosts with both membrane faces exposed (data not shown). The IOV preparation is composed mainly of intact inside-out vesicles (29; also this work).

Initial cross-linking attempts with these preparations revealed a multiplicity of labeled products (Fig. 1a). Inclusion of two washing steps after the incubation but prior to photolysis reduced the number of bands formed (Fig. 2). The labeled products represent Ca\(^{2+}\)-dependent, CaM-specific cross-links, since their formation was blocked by inclusion of EGTA in the reaction mixture, or preincubation with a large excess of unmodified calmodulin. By these criteria, bands at apparent M\(_s\) = 168,000 in the I\(_{50}\) and in the IOV did appear to represent specific labeling. Other products were observed, but none appeared in both I\(_{50}\) and IOV (Table I).

In order to demonstrate the identity between the major cross-linked product and the (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase pump, a correlation between cross-linked product formation and enzymatic function was sought. The extent of product formation is dependent upon photolysis time (Fig. 3). An exposure of 4
min yielded maximum product (Fig. 3) while not damaging the ATPase function (Table II).

Andreasen et al. (27) have shown that azido-CaM was essentially as effective as unmodified calmodulin in activating the CaM-stimulated phosphodiesterase from bovine heart. However, the focus of that study was to demonstrate the ability of azido-CaM to form active noncovalent associations with a calmodulin-regulated enzyme. In the present work we wished to look for active covalent CaM-ATPase complexes. There is no a priori reason to assume that the photocross-linked product either would or would not retain an active conformation. The data in Table II suggest that the cross-link does occur with retention of enzymatic function. When incubated with increasing azido-CaM or azido-\(^{125}\)I-CaM concentrations, the photolyzed \(I_{50}\) membranes exhibited increasing basal ATPase activities. The ATPase was capable of being further stimulated to the same maximal activity, indicating that few nonactive cross-links were formed. Quantitation of the \(I_{50}\) counts incorporated into the 168,000-dalton product demonstrated a correlation between amount of product formed and amount of basal activity increase (Fig. 4). The binding and activity increase data can be extrapolated to give an estimate of 180 ATPase sites/RBC \(I_{50}\) ghost, based upon \(6.6 \times 10^{-13}\) g of protein/10^9 RBC equivalent (41). Previous reports for total membrane (\(Ca^{2+} + Mg^{2+}\))-ATPase sites are 400 (42) and 700 (43).

IOV can be used to study \(Ca^{2+}\) pump activity by measuring transport of \(Ca^{2+}\) into the vesicles. Photolysis of IOV incubated with increasing azido-\(^{125}\)I-CaM concentrations yielded increased basal \(Ca^{2+}\) transport rates (Fig. 5B). Quantitation of the \(I_{50}\) counts in the 168,000-dalton cross-link product indicated a correlation between amount of product formed and amount of basal transport increases. Using a value of \(4.5 \times 10^{-13}\) g of protein/IOV-RBC membrane equivalent, a double reciprocal plot gave a total binding estimate of 1,600 azido-\(^{125}\)I-CaM binding sites/RBC-IOV ghost. Other investigators have reported considerably higher total CaM binding values, e.g., 11,000 (calculated from the data of Ref 44 and the protein/ghost value of \(6.6 \times 10^{-13}\)) (41), 4,100 (45), and 4,500 (46).

The differences in the IOV CaM binding sites and \(I_{50}\) ATPase sites observed in this study in contrast to other studies cannot readily be explained. However, from the autoradiogram of IOV and \(I_{50}\) membranes it is apparent that the number and pattern of CaM binding proteins is dependent upon the type of membranes used. Several proteins are able to form cross-linked products with azido-\(^{125}\)I-CaM in a \(Ca^{2+}\)-dependent manner. All product formation was blocked by prior incubation of the membranes with unmodified calmodulin. The major differences between the membranes used in the present study are the intactness of the IOV, and the presence of the spectrin network in the \(I_{50}\) vesicles. Spectrin bands can clearly be seen in the Coomassie brilliant blue-stained gels of \(I_{50}\) membranes but not for IOV membranes (Fig. 1b). The appearance of the lower molecular weight cross-linked products could be due to limited proteolysis, if differential susceptibility or exposure to proteases exists between \(I_{50}\) and IOV, or to fortuitous nearest neighbor cross-links. It is possible that the accessibility of some membrane proteins to calmodulin is dependent upon the state of the membrane. Thus, these cross-linked products could represent specific and real interactions of azido-\(^{125}\)I-CaM with smaller membrane proteins. The physiological significance of these associations is open to question.

While we have not attempted to identify those cross-linked products unique to each membrane type, we believe that the products of apparent \(M_r = 168,000\) in \(I_{50}\) and IOV represent a specific, \(Ca^{2+}\)-dependent cross-linked between calmodulin and the \((Ca^{2+} + Mg^{2+})\)-ATPase pump. Since azido-\(^{125}\)I-CaM migrates with an apparent \(M_r\) of 20,000 in our gels (1.9 mm EDTA is present in the SDS sample buffer), the calmodulin binding capacity of the ATPase pump resides in a polypeptide of about 150,000 daltons. The molecular weight of the cross-linked product and the lack of significant amount of appropriate higher molecular weight product suggests a CaM-ATPase binding stoichiometry of 1:1. Together with previous studies indicating a 1:1 complex formation between calmodulin on other binding proteins, these results suggest that a 1:1 binding stoichiometry is the general rule in \(Ca^{2+}\)-dependent calmodulin-regulated systems. The ability of azido-\(^{125}\)I-CaM to cross-link with retention of native activity, together with the inherent sensitivity of the autoradiographic technique indicates that this agent may not only be useful in probing the molecular basis for the calmodulin-ATPase interaction but may also be of general utility in defining additional roles of calmodulin in the erythrocyte.

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