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Staining of the Ca^{2+}-binding Proteins, Calsequestrin, Calmodulin, Troponin C, and S-100, with the Cationic Carbocyanine Dye "Stains-all"*

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The Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, have all been shown to stain dark blue or purple with the cationic carbocyanine dye "Stains-all", while most proteins stain red or pink. Stains-all staining of these Ca^{2+}-binding proteins was 50–100% more intense than Coomassie blue staining, and the blue stained calsequestrin and troponin C could be detected in muscle extracts. Densitometric scans of Stains-all-stained gels revealed that interaction of the dye with Ca^{2+}-binding proteins changed the absorption spectrum of the dye. The dye-protein complex absorbed maximally at 615 nm, reflecting a state that results from the binding of individual dye molecules at anionic sites. Stains-all also interacted with undenatured Ca^{2+}-binding proteins in aqueous solution forming a complex absorbing maximally at 600 nm. A simple assay for Ca^{2+}-binding proteins has been developed using this property. These results suggest that the interaction of the dye with anionic sites within these Ca^{2+}-binding proteins produces the dye-protein complex which absorbs at 600–615 nm. Stains-all staining of these Ca^{2+}-binding proteins will be useful in their identification and purification from various tissues. It might also be a valuable tool in the identification of potential Ca^{2+}-binding proteins.

"Stains-all", a cationic carbocyanine dye, has been shown to stain sialoglycoproteins and phosphoproteins blue and almost all other proteins red (1–4). Interaction of Stains-all with the different macromolecules causes changes in the absorption spectrum of the dye, resulting in the different colored dye-protein complexes (5, 6). At least five different complex states of polymer-dye interaction have been found (5, 6). The complex states and corresponding spectral band maxima are α state (510 nm), β state (535 nm), γ states (500–510 nm), J state (610–650 nm), $\beta\alpha$ state (550 nm), and S state (470 nm) (5, 6). Each state depends on the adsorption of the dye by the macromolecule and on the nature and conformation of the macromolecule. Blue staining (J state, 610–650 nm) results from the interaction of individual dye molecules at anionic sites such as sialic acid or phosphoryl groups in the protein (1–4). Removal of the anionic portions leads to a loss of the blue complex (1–4).

Jones *et al.* (7) and Jones and Cala (8) found that several proteins associated with cardiac sarcoplasmic reticulum stained blue or purple with Stains-all. We recently purified one of these proteins and showed that it was the cardiac form of calsequestrin (9). We also found that purified calsequestrin from several different muscle sources stained blue with Stains-all and that all of the blue-staining proteins from both skeletal and cardiac sarcoplasmic reticulum would bind to calcium phosphate after extraction from the membrane (9). Attempts to rationalize the blue staining of calsequestrin has led us to the finding that several well known Ca^{2+}-binding proteins stain blue with Stains-all.

EXPERIMENTAL PROCEDURES

Materials—SDS,[1] acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and TEMED were purchased from Bio-Rad. The cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethylnaphtho [1, 2d] thiazolin-2-ylidene)-2-methylpropenyl] naptho [1,2d] thiazolium bromide] was obtained from Eastman Organic Chemicals and prepared as a 0.1% stock solution in formamide. S-100 and calmodulin were purified from bovine brain and were obtained from Calbiochem-Behring. Calcineurin purified from bovine brain was obtained from Boehringer Mannheim. Rabbit skeletal muscle troponin C, troponin I, and troponin T were generous gifts from Dr. Richard Ingraham and Dr. Charles A. Swenson, University of Iowa. Human red cell ghosts were a generous gift from Stephen Merfeld and Dr. Michael L. Jennings, University of Iowa. *Staphylococcus aureus* V8 protease was obtained from Pierce Chemical Co.

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of MacLennan (10) as modified by Campbell and MacLennan (11). Muscle extracts are defined as the supernatant from a rabbit muscle homogenate following centrifugation at 10,000 × *g* for 20 min. Skeletal muscle calsequestrin was purified using the calcium phosphate precipitation method described for cardiac calsequestrin (9). Degraded calsequestrin was obtained by incubating partially purified calsequestrin at room temperature overnight. Protein was determined by the method of Lowry *et al.* (13) using bovine serum albumin as a standard.

Gel Electrophoresis—The analysis of proteins by SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (14) in 1.5-mm thick gradient gels (5–15 or 8–18% acrylamide). Usually 100–200 μg of membrane protein or 5–15 μg of purified protein were applied per gel lane. Apparent molecular weights were calculated from a graph of relative mobilities *versus* log molecular weight for standard proteins. Two-dimensional gel electro-

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

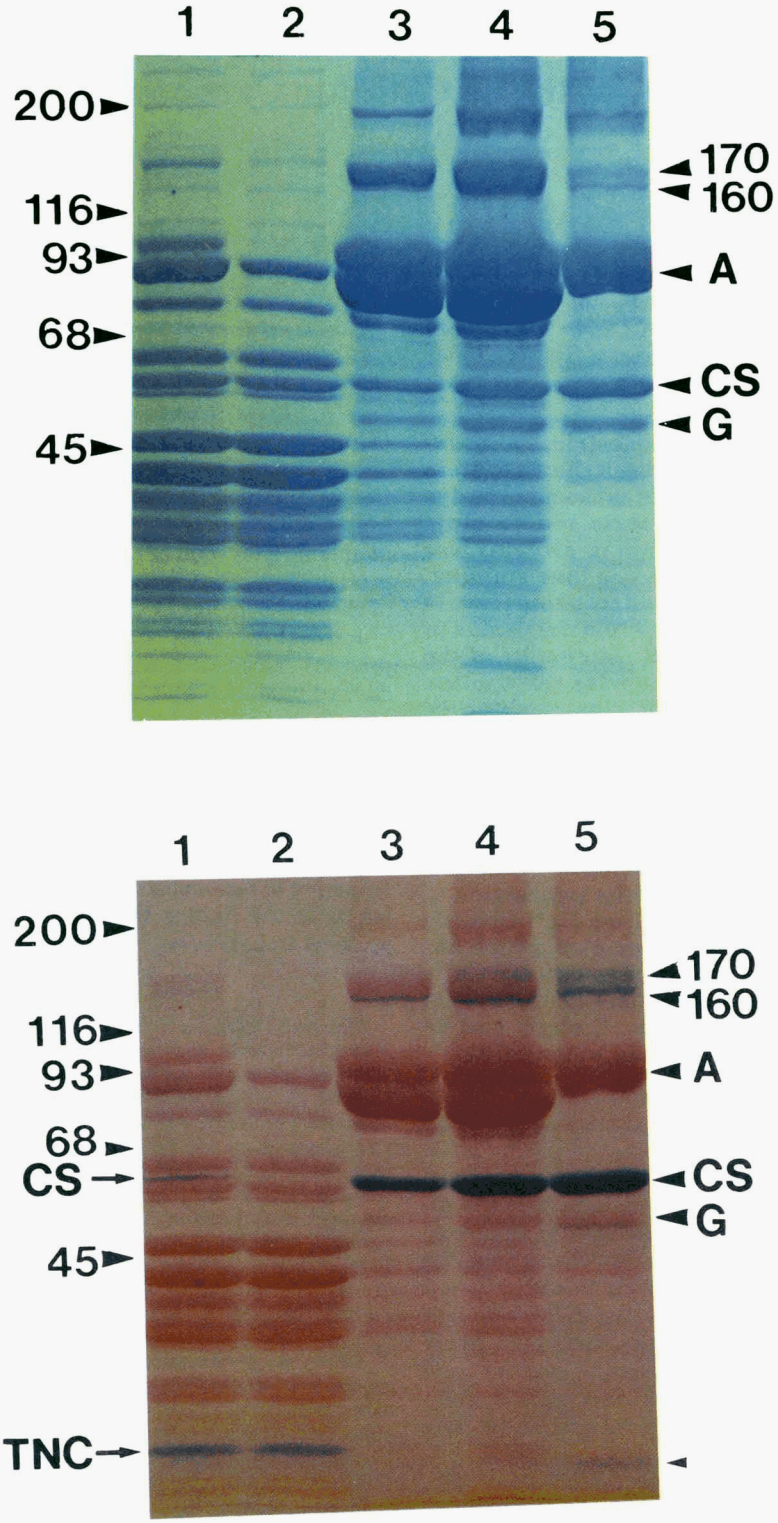

FIG. 1. **Coomassie blue and Stains-all staining of skeletal muscle proteins.** Fractions in the purification of sarcoplasmic reticulum vesicles from rabbit skeletal muscle according to Ref. 11 were analyzed by SDS-polyacrylamide gel electrophoresis (5–15% gradient gels) and stained with Coomassie blue (*top*) or Stains-all (*bottom*) as described under "Experimental Procedures." *Lane 1*, supernatant from rabbit skeletal muscle homogenate following centrifugation at 10,000 × *g* for 20 min; *lane 2*, supernatant from rabbit skeletal muscle homogenate following centrifugation at 50,000 × *g* for 1 h; *lane 3*, pellet obtained from 50,000 × *g* centrifugation; *lane 4*, supernatant following 7,000 × *g* centrifugation; *lane 5*, KCl-washed sarcoplasmic reticulum vesicles. Coomassie blue-stained gel contained 100 μg of protein in each lane, while the Stains-all-stained gel contained 200 μg of protein in each lane. *A*, (Ca^{2+} + Mg^{2+})-ATPase (M_r = 105,000); *CS*, calsequestrin (M_r = 63,000); *G*, glycoprotein (M_r = 53,000); *160*, 160,000-Da glycoprotein; *170*, 170,000-Da protein; *TNC*, troponin C. The *numbers* on the *left side* represent molecular weight × 10^{-3} of molecular weight standards. The *small arrowhead* indicates a lower molecular weight blue-staining protein.

phoresis was carried out as previously described (15). Cleveland peptide maps (16) were performed with *S. aureus* V8 protease. The effect of Ca^{2+} on the electrophoretic mobility of the blue-staining sarcoplasmic reticulum proteins or other Ca^{2+}-binding proteins was analyzed according to the method of Klee *et al.* (17).

Stains-all Staining of Ca^{2+}-binding Proteins—Staining with the cationic carbocyanine dye Stains-all was carried out as described (1) with the following modifications. Slab gels were fixed overnight with 25% isopropyl alcohol and washed exhaustively in 25% isopropyl alcohol to remove SDS. The gels were then stained in the dark for at least 48 h with 0.0025% Stains-all, 25% isopropyl alcohol, 7.5% formamide, and 30 mM Tris base, pH 8.8. This staining procedure has been optimized for the blue-staining Ca^{2+}-binding proteins. We found that use of the original staining procedure described by King and Morrison (1) resulted in blue staining of calsequestrin but other Ca^{2+}-binding proteins stained red. Slab gels could be stained with Coomassie blue following Stains-all staining if they were destained completely in 25% isopropyl alcohol. Stains-all-stained gels and Coomassie blue-stained gels were scanned using a Gilford spectrophotometer and gel scanner.

Stains-all Binding to Native Ca^{2+}-binding Proteins in Aqueous Solution—The interaction of Stains-all with various Ca^{2+}-binding proteins was also studied in aqueous solution. The standard solution contained 10 mM Tris base, pH 8.8, 0.001% Stains-all, and 0.1% formamide. Ca^{2+}-binding proteins (0.5–12 μg) were added to 1.0 ml of solution and then incubated at room temperature in the dark for 30 min. The absorbance at 600 nm was then measured against a control solution, containing no protein, using a Gilford spectrophotometer.

RESULTS

Various fractions during the purification of sarcoplasmic reticulum vesicles from rabbit skeletal muscle homogenates have been analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue and Stains-all (Fig. 1). Almost all of the proteins found in skeletal muscle extracts, including the (Ca^{2+} + Mg^{2+})-ATPase and the 53,000-Da glycoprotein of the sarcoplasmic reticulum, stained red or pink with Stains-all (Fig. 1, *bottom*). Two proteins in the skeletal muscle extract stained blue with Stains-all (Fig. 1, *bottom, lane 1*). One was identified as calsequestrin by its mobility and by the fact that it purified with the sarcoplasmic reticulum vesicles (Fig. 1, *bottom, lane 5*). The second protein was present in both the homogenate (Fig. 1, *bottom, lane 1*) and the supernatant following removal of the sarcoplasmic reticulum vesicles (Fig. 1, *bottom, lane 2*). It had a mobility similar to troponin C. The 160,000-Da glycoprotein and a 170,000-

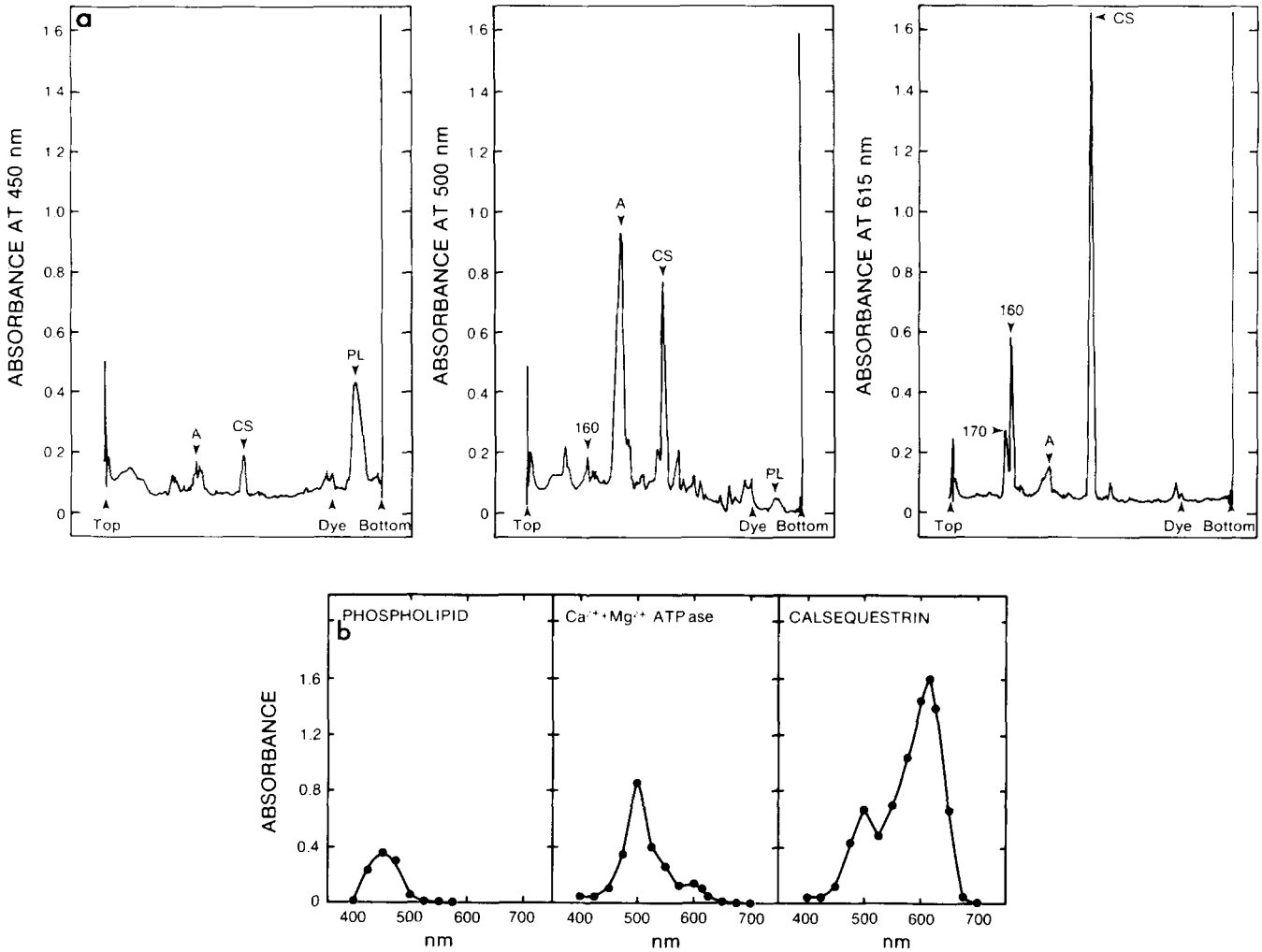

FIG. 2. **Densitometric scans (*a*) and spectral absorbance curves (*b*) of sarcoplasmic reticulum proteins stained with Stains-all.** *a*, densitometric scans of sarcoplasmic reticulum proteins separated by Laemmli (14) SDS-gel electrophoresis (5–15% acrylamide) were performed on a Gilford spectrophotometer at 450, 500, and 615 nm. The top and bottom of the gel are indicated along with the position of the bromphenol blue dye front. *A*, (Ca^{2+} + Mg^{2+})-ATPase (M_r = 105,000); *CS*, calsequestrin (M_r = 63,000); *160*, 160,000-Da glycoprotein; *170*, 170,000-Da protein, *PL*, phospholipid. *b*, sarcoplasmic reticulum proteins were analyzed on SDS gels as described in *a*. The Stains-all-stained gels were scanned at various wavelengths (400–700 nm), and absorbance for each macromolecule (phospholipid, (Ca^{2+} + Mg^{2+})-ATPase, and calsequestrin) was plotted for each wavelength.

Da protein of the sarcoplasmic reticulum both stained blue with Stains-all and were enriched in the KCl-washed sarcoplasmic reticulum (Fig. 1, *bottom, lane 5*). In crude membrane preparations (Fig. 1, *bottom, lanes 3* and *4*), the 160,000-Da glycoprotein and 170,000-Da protein had mobilities similar to the subunits of phosphorylase kinase (145,000 and 130,000 Da) and glycogen-debranching enzyme (160,000 Da) all of which stained red with Stains-all. KCl washing, which removed the glycolytic enzymes from the sarcoplasmic reticulum vesicles, did not remove the 160,000- and 170,000-Da blue-staining proteins, showing that they were not related to phosphorylase kinase or glycogen-debranching enzyme. A low molecular weight blue-staining protein which had the same mobility as calmodulin and may have been calmodulin was also seen in the KCl-washed vesicles.

In order to quantitate the Stains-all staining of the sarcoplasmic reticulum proteins, we performed densitometric scans of the Stains-all-stained gels (Fig. 2, *a* and *b*). The (Ca^{2+} + Mg^{2+})-ATPase and the 53,000-Da glycoprotein stained red and absorbed maximally at 500 nm (Fig. 2, *a* and *b*). Calsequestrin, the 160,000-Da glycoprotein, and 170,000-Da protein stained blue with Stains-all and absorbed maximally at 615 nm (Fig. 2, *a* and *b*). Phospholipids, which ran in front of the bromphenol blue dye front, stained yellow-orange and absorbed maximally at 450 nm (Fig. 2, *a* and *b*). Spectral absorbance curves were taken at 25-nm intervals from 400 to 700 nm for phospholipid, the (Ca^{2+} + Mg^{2+})-ATPase, and calsequestrin (Fig. 2*b*). Phospholipid had an absorption maxima at 450 nm. The stained (Ca^{2+} + Mg^{2+})-ATPase had an absorption maxima at 500 nm and a small shoulder at 600 nm. Calsequestrin had an absorption maxima at 615 nm and a second smaller peak at 500 nm. The binding of Stains-all to calsequestrin thus resulted in a dye-calsequestrin complex which absorbed maximally at 615 nm and corresponded to the J complex of the dye. This complex has been shown to result from the interaction of individual dye molecules at anionic sites (5, 6). Therefore, the blue staining of calsequestrin would appear to result from Stains-all binding to anionic sites within calsequestrin.

In order to investigate the location of the anionic sites in calsequestrin which were responsible for the blue staining, we carried out Cleveland peptide maps (16) of purified skeletal muscle calsequestrin (Fig. 3). Calsequestrin digestion required very low levels of *S. aureus* V8 protease, probably because of its high aspartic and glutamic acid content. All of the peptide fragments of calsequestrin that were detected with Coomassie blue stained blue with Stains-all. Since Stains-all staining of calsequestrin was more sensitive than Coomassie blue staining, several peptides could be seen with Stains-all that were not detected with Coomassie blue (Fig. 3). Thus, Stains-all must have interacted with several different anionic sites within the calsequestrin molecule. Similar results have been obtained with cardiac calsequestrin (data not shown). Since calsequestrin does not contain either sialic acid or phosphate (12), the only anionic sites which could bind Stains-all are acidic amino acid residues contained within calsequestrin.

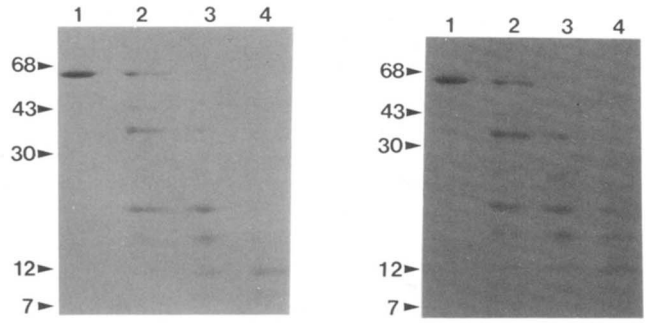

FIG. 3. **Cleveland peptide maps of skeletal muscle calsequestrin stained with Coomassie blue or Stains-all.** Purified calsequestrin was electrophoresed on SDS gels and excised following a brief staining with Coomassie blue. The gel slices containing equal amounts of calsequestrin were then placed in sample wells of a second 13.5% acrylamide gel. The slices were then overlayed with 0, 5, 20, and 100 ng of Staph A protease in *lanes 1–4*, respectively. Digestion proceeded in the stacking gel and during subsequent electrophoresis, and the gels were then stained with either Coomassie blue (*left*) or Stains-all (*right*) as described under "Experimental Procedures." The *numbers* on the *left side* represent molecular weight × 10^{-3} of molecular weight standards.

Endo-β-*N*-acetylglucosaminidase H digestion of rabbit muscle sarcoplasmic reticulum results in the removal of high mannose oligosaccharide chains from the 53,000- and 160,000-Da glycoproteins and lowers their apparent molecular mass to 49,000 and 155,000 Da, respectively (11). We have previously shown that the Stains-all blue-staining band of 160,000 Da was shifted to 155,000 Da following endo-β-*N*-acetylglucosaminidase H digestion and that Stains-all blue staining remained after carbohydrate removal (9). Endo-β-*N*-acetylglucosaminidase H removal of the carbohydrate from canine cardiac calsequestrin or from the 130,000-Da cardiac glycoprotein did not affect the Stains-all blue staining of either of these proteins. These observations confirm that cardiac calsequestrin and the higher molecular weight glycoproteins of skeletal and cardiac sarcoplasmic reticulum stained blue with Stains-all because of binding of the dye to the protein portion of the molecule rather than to the carbohydrate portion. Stains-all blue staining was also not correlated with protein acidity because all of the proteins which eluted from DEAE-cellulose with greater than 200 mM salt stained red or pink except for calsequestrin and the higher molecular weight glycoproteins.

We also examined the relationship between blue staining and Ca^{2+} binding. We have already shown that Stains-all will stain the low affinity Ca^{2+}-binding protein calsequestrin regardless of the muscle source (9). Therefore, we decided to investigate the Stains-all staining properties of a series of high affinity Ca^{2+}-binding proteins. Fig. 4, *top*, *lanes 4–8*, shows that calsequestrin, degraded calsequestrin, calmodulin, troponin C, and S-100 stained deep blue or purple with Stains-all. This is in contrast to serum proteins (*lane 1*), red cell proteins except glycophorin (*lane 2*), or a series of molecular weight standards (*lane 9*) all of which stained red or pink with Stains-all. One red cell protein stained blue with Stains-all, and it ran with a mobility that corresponded to glycophorin. Therefore, our modified staining procedure still stained sialic acid-rich proteins blue. It has been shown that glycophorin will stain red following removal of the sialic acid residues (1). Troponin I and troponin T also stained red with Stains-all (data not shown). Calcineurin B, which binds Ca^{2+} with high affinity, also stained blue with Stains-all, while calcineurin A, which does not bind Ca^{2+}, stained red (data not shown). Spectral absorbance curves for calmodulin, troponin C, and S-100 stained with Stains-all are shown in Fig. 4, *bottom*. Calmodulin and troponin C both had absorption maxima at 615 nm, while S-100 (which stained purple) had equal absorption maxima at 500 and 615 nm. Thus, the blue staining of these high affinity Ca^{2+}-binding proteins also resulted from Stains-all binding to anionic sites.

The ratio of Stains-all staining (measured at 615 nm) to that of Coomassie blue staining (measured at 575 nm) was 1.3 for calsequestrin, 2.0 for calmodulin, 1.4 for troponin C, and 2.2 for S-100. Therefore, in addition to differentially staining these Ca^{2+}-binding proteins blue, Stains-all is a more

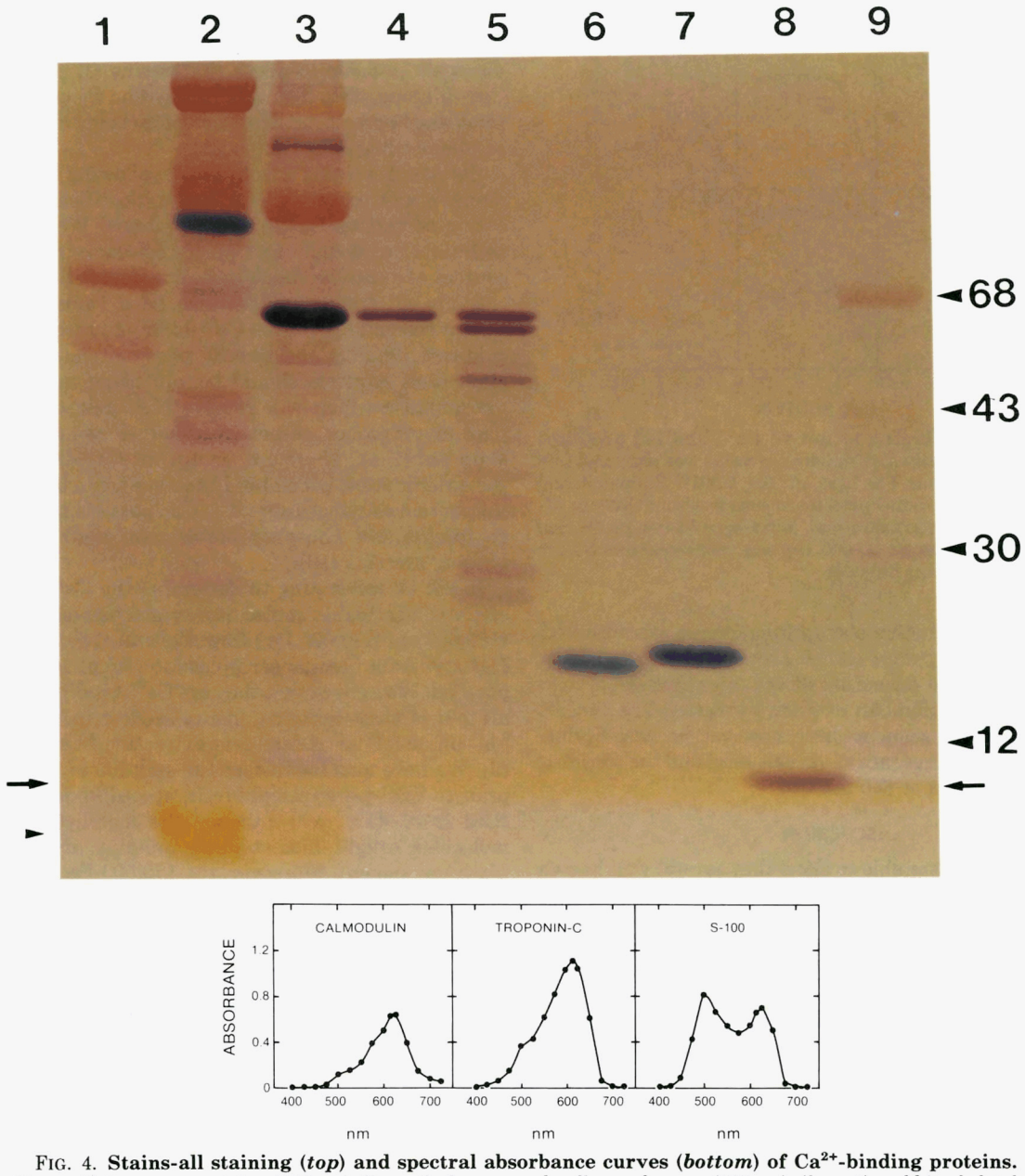

FIG. 4. **Stains-all staining (*top*) and spectral absorbance curves (*bottom*) of Ca^{2+}-binding proteins.** *Top*: rabbit serum proteins (200 μg) (*lane 1*), human red cell membranes (200 μg) (*lane 2*), rabbit skeletal sarcoplasmic reticulum vesicles (200 μg) (*lane 3*), purified rabbit skeletal muscle calsequestrin (M_r = 63,000; 4 μg) (*lane 4*), degraded calsequestrin (4 μg) (*lane 5*), calmodulin (M_r = 19,000; 12 μg) (*lane 6*), troponin C (M_r = 19,500; 12 μg) (*lane 7*), S-100 (12 μg) (*lane 8*), and molecular weight standards: bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), cytochrome *c* (12,000) (*lane 9*) were analyzed on Laemmli (14) SDS-slab gels (8–18% gradient of acrylamide) and stained with Stains-all as described under "Experimental Procedures." *Numbers* on the *right side* represent molecular weight × 10^{-3}. The dye front is indicated by *arrows*. Yellow-staining lipids (*lanes 2* and *3*) are indicated by the *small arrowhead*. *Bottom*: purified proteins were analyzed on SDS gels, stained with Stains-all and scanned as described in Fig. 2. Absorbance of calmodulin (12 μg), troponin C (14 μg) and S-100 (12 μg) stained with Stains-all was plotted for each wavelength.

sensitive stain for these Ca^{2+}-binding proteins than is Coomassie blue.

Since Stains-all staining was carried out after SDS-gel electrophoresis, we were actually seeing the interaction of the dye with the SDS-denatured Ca^{2+}-binding proteins. In order to determine the interaction of Stains-all with native Ca^{2+}-binding proteins, we measured the absorption spectrum of Stains-all in the presence of various Ca^{2+}-binding proteins in aqueous solution. Stains-all absorbs maximally at 525 nm, but the addition of any of the Ca^{2+}-binding proteins caused a shift in the absorption spectra to approximately 600 nm. This indicates that the dye will form a similar complex with the native Ca^{2+}-binding proteins as it did with SDS-denatured proteins. We used this shift in absorption spectra to determine the formation of the dye-protein complex. The absorbance of this complex at 600 nm was measured against a control dye solution, and the difference was plotted *versus* the amount of protein added for each Ca^{2+}-binding protein (Fig. 5). The absorbance at 600 nm increased linearly for each Ca^{2+}-binding protein (0.5–4 μg) and then leveled off with increasing amounts of protein (Fig. 5). The range of the linear relationship between absorbance and protein concentration depended on the dye concentration in the solution. Bovine serum albumin, which stained red, gave little absorption until 4 μg,

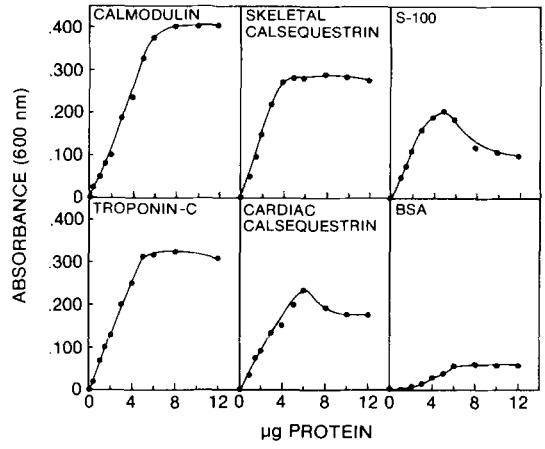

FIG. 5. **Stains-all binding to native Ca^{2+}-binding proteins.** Stains-all binding to native Ca^{2+}-binding proteins was measured in a solution containing 10 mM Tris base, pH 8.8, 0.001% Stains-all, and 0.1% formamide. Ca^{2+}-binding proteins or bovine serum albumin (0–12 μg) were added to this solution, and, following a 30-min incubation in the dark, the absorbance at 600 nm was measured against the control solution containing only dye.

and from 8–12 μg no further absorption increase was observed. We have preliminary data showing that Ca^{2+} will compete with Stains-all in the formation of the dye-calmodulin complex in aqueous solution. An extensive investigation of Ca^{2+} competition does not seem possible, however, because Stains-all aggregates in the presence of various salts and the resulting dye aggregate absorbs at 650 nm (5).

DISCUSSION

In this study we were able to show that several well known high or low affinity Ca^{2+}-binding proteins could be easily identified by their blue staining in the presence of Stains-all. Our findings are consistent with the interpretation that Stains-all binds to anionic sites within Ca^{2+}-binding proteins and the resulting J complex gives rise to the blue-staining property. Previous studies have shown that Stains-all stains sialoglycoproteins (*i.e.* glycophorin) and phosphoproteins (*i.e.* phosvitin and casein) blue while staining most proteins red or pink (1–4). The blue staining of these proteins resulted from the complex formed between the dye and the anionic sites created by sialic acid residues or phosphoryl groups in these proteins (1–4).

Although our observations support the view that Ca^{2+}-binding proteins will stain blue with Stains-all, it is probable that the extent of blue staining is dependent on the number of Ca^{2+}-binding sites in the protein. Calsequestrin has one Ca^{2+}-binding site/9 amino acid residues, and troponin C and calmodulin have about one Ca^{2+}-binding site/40 amino acid residues; all these proteins stain blue. By contrast, the (Ca^{2+} + Mg^{2+})-ATPase (105,000 Da) has one Ca^{2+}-binding site/500 residues and stains red. The small shoulder at 600 nm which is seen in the scan of the Stains-all-stained ATPase (Fig. 2*b*) might represent Stains-all binding to the anionic Ca^{2+}-binding sites in the ATPase, but the predominant interactions between Stains-all and the remainder of the ATPase molecule results in red staining. The Ca^{2+}-binding sites in calmodulin and troponin C are EF hands (18), whereas the Ca^{2+}-binding sites in calsequestrin are clearly not of sufficiently high affinity to be EF hands. Nevertheless, both types of protein stain blue. Therefore, the blue staining is not related to a specific type of Ca^{2+}-binding site.

The formation of the J complex with Ca^{2+}-binding proteins in aqueous solution indicates that Stains-all will also bind to the anionic sites in the undenatured Ca^{2+}-binding proteins. Due to the aggregation of the dye with various salts, it does not seem possible to prove that Stains-all is binding to the Ca^{2+}-binding sites. The dye aggregation by salt requires that samples of protein are desalted before being added to aqueous solutions containing Stains-all.

Stains-all staining in solution or following SDS-gel electrophoresis will be very useful in the identification and purification of various Ca^{2+}-binding proteins. We have used this technique to identify and purify calsequestrin from several sources of excitable tissue including bovine brain.[2]

Stains-all staining might also be a valuable tool in the identification of potential Ca^{2+}-binding proteins. Since sialic acid-rich proteins and heavily phosphorylated proteins also stain blue, controls should be run using neuraminidase or phosphatase which will remove the sialic acid residues or phosphoryl groups, respectively, and these proteins will then stain red (1–4). Therefore, except for Ca^{2+}-binding proteins, the anionic sites, which bind Stains-all, can be removed from the protein enzymatically. It is also possible that the sialoglycoproteins and phosphoproteins that stain blue are Ca^{2+}-binding proteins (19).

It will be interesting to determine the Ca^{2+}-binding properties of the higher molecular weight blue-staining proteins (160,000 and 170,000 Da) from skeletal (130,000 and 140,000 Da) and from cardiac sarcoplasmic reticulum once they are purified. We believe that they are Ca^{2+}-binding proteins since all four of these proteins, like calsequestrin, bind to calcium phosphate following detergent extraction from the membrane (9). We have also found that the addition of Ca^{2+} to samples prior to SDS-gel electrophoresis, according to the method of Klee *et al.* (17), will enhance the mobility of the higher molecular weight blue-staining proteins and calsequestrin (data not shown). Moreover, the 170,000-Da protein in skeletal sarcoplasmic reticulum has a shift in apparent molecular weight (170,000–150,000 Da) depending on the pH of the electrophoresis buffers (data not shown). This is the same type of mobility change which was observed for calsequestrin (9). All of these data strongly suggest that the higher molecular weight blue-staining proteins are Ca^{2+}-binding proteins like calsequestrin.

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