Isolation of a High Affinity Calcium-binding Protein from Sarcoplasmic Reticulum*

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SUMMARY

Seven proteins have been isolated from sarcoplasmic reticulum. These proteins have been identified as an ATPase, a series of water soluble, acidic proteins of molecular weights 55,000, 46,500, 38,000, 33,000, and 20,000, and a proteolipid. The interactions of the acidic proteins with Ca** have been measured at pH 7.5 in the presence and absence of 0.1 M KCl. The protein of molecular weight 55,000 is the only protein with high affinity for Ca**. In the presence of 0.1 M KCl it binds 16 to 22 nmoles of Ca** per mg (about 1 mole of Ca** per mole) with a dissociation constant between 2.5 and 4 pM. It also binds several hundred nanomoles of Ca** with a dissociation constant greater than 5,000 pM. In the absence of KCl the protein of molecular weight 55,000 displays the high affinity Ca** binding site and sites which bind about 400 nmoles of Ca** per mg with a dissociation constant of about 120 pM. In the presence of 0.1 M KCl, calsequestrin (mol wt 46,500) does not show high affinity Ca** binding but binds about 850 nmoles of Ca** per mg with a dissociation constant of about 800 pM. In the absence of KCl, calsequestrin binds about 900 nmoles of Ca** per mg with a dissociation constant of about 60 pM. In the absence of KCl the acidic proteins with molecular weights between 20,000 and 38,000 bind from 90 to 1,100 nmoles of Ca** per mg with dissociation constants in the range of 115 to 150 pM. Apparent high affinity Ca** binding is observed in these fractions in the absence of KCl but, in the presence of 0.1 M KCl, both the apparent high affinity Ca** binding and the low affinity binding are greatly reduced.

The sarcoplasmic reticulum of skeletal muscle controls the concentration of cytoplasmic Ca**, thereby controlling contraction and relaxation of the muscle (1, 2). During relaxation, calcium is pumped from the cytoplasm and stored within the sarcoplasmic reticulum; upon excitation calcium is released to the cytoplasm where it binds to troponin and releases inhibition of the actin-myosin interaction. The sarcoplasmic reticulum has been isolated in pure form and has been shown to have only one major activity, a Ca**-dependent ATPase, and one major function, Ca** binding and release (3).

The binding of Ca** by sarcoplasmic reticulum can be studied in several ways. In the presence of ATP, a limited amount of Ca**, about 150 nmoles per mg of protein, is bound to membranous sites of sarcoplasmic reticulum (4, 5). The membranous sites appear to be internal organic anions which make up part of the membrane itself. In the presence of ATP and a permeant anion, such as phosphate or oxalate, several micromoles of Ca** per mg of protein can be precipitated within the sarcoplasmic reticulum vesicles (6). Rates of transport across the membrane can be estimated by use of this procedure. A third approach is to measure passive binding of Ca** by membranes exposed to various Ca** concentrations. In this way sites of high and low affinity on undefined sites of the vesicles can be detected. Chevallier and Butow (7) have found that about 130 nmoles of Ca** are bound per mg of protein with a dissociation constant of 13 pM and that 10 to 20 nmoles of Ca** are bound per mg with a dissociation constant of 0.13 pM. Cohen and Selinger (8) report passive binding of 50 nmoles of Ca** per mg of protein with a dissociation constant of 40 pM and Meissner et al. (9) report that sarcoplasmic reticulum binds 10 to 15 nmoles of Ca** per mg with a dissociation constant of 1 pM.

A fourth way to study Ca** binding is by measuring the Ca** binding ability of proteins isolated from sarcoplasmic reticulum. In a series of publications (10-13) we have shown that sarcoplasmic reticulum proteins can be fractionated into an intrinsic protein fraction consisting of an ATPase, phospholipid and proteolipid, and an extrinsic protein fraction consisting of a series of water-soluble acidic proteins. The ATPase has active sites equivalent to 10 nmoles per mg of protein and these sites can account for binding of 10 nmoles of Ca** per mg with a dissociation constant of 0.1 to 1 nM (9, 11). The acidic protein fraction can account for a great deal more Ca** binding (12). We have described the purification and properties of calsequestrin, a protein with high capacity, medium affinity Ca** binding (12). This observation has now been confirmed in two other laboratories (9, 14). We have also given a preliminary description of Ca** binding by two other acidic protein fractions (13). In this communication we describe more fully the Ca**-binding ability of the various acidic proteins. One of the water-soluble proteins...
has the ability to bind 1 mole of Ca\(^{2+}\) per mole with a dissociation constant between 2 and 4 \(\mu M\).

**MATERIALS AND METHODS**

**Preparation of Acidic Proteins**

A. **High Affinity Calcium-binding Protein**

*Step 1*—Sarcoplasmic reticulum was prepared from back and leg muscles of 3.5-kg rabbits as described earlier (10). The R1 (washed) fraction was used in all preparations. Sarcoplasmic reticulum was suspended at a protein concentration of 25 mg per ml in Buffer A (a solution containing 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.0, and 1 mM histidine). The suspension was made 1 M in KCl by the addition of solid KCl and 1 mM in diithiothreitol by the addition of a 0.1 M solution. Deoxycholate (10% w/v; pH 8.0) was added with stirring to give a final concentration of 0.1 mg of deoxycholate per mg of protein. After incubation for 10 min at 4\(^{\circ}\), the suspension was centrifuged at 165,000 \(\times\) g for 30 min. The opalescent supernatant was removed with a Pasteur pipette; care being taken not to remove any of the loosely packed pellet. The deoxycholate extract was dialyzed first for 4 hours and then for 18 hours, at 4\(^{\circ}\) against 3 liters of Buffer B (a solution containing 5 mM Tris-HCl, pH 7.5, and 0.1 mM ethylene glycol bis(\(\beta\)-aminoethyl ether)-N\(^\cdot\)N\(^\cdot\)tetracetic acid). At 4 and 22 hours insoluble protein was removed by centrifugation at 165,000 \(\times\) g for 30 min. The opalescent supernatant was added with stirring to give a final concentration of 0.1 mg of deoxycholate per mg of protein. After incubation for 10 min at 4\(^{\circ}\), the suspension was centrifuged at 165,000 \(\times\) g for 30 min.

*Step 2*—The supernatant solution in 35- to 40-ml portions was passed through a column of DEAE-cellulose (1.5 \(\times\) 18 cm) equilibrated with Buffer C (a solution of 5 mM Tris-HCl, pH 7.5) and the column was eluted with 400 ml of Buffer C that contained a linear gradient of KCl from 0 to 0.7 M. The first protein peak containing phosphorylase and other nonreticulum proteins was discarded. The second shallow peak, eluted at a KCl concentration of about 0.33 M, contained the high affinity calcium-binding protein. The third peak, eluted at a KCl concentration of about 0.48 M, was saved as a source of calsequestrin and the 20,000 to 38,000 molecular weight proteins.

*Step 3*—Protein in the second peak was concentrated by ultrafiltration under pressure (Amicon Diaflo apparatus) and dialyzed overnight against Buffer D (a solution of 0.15 M KCl, 0.01 M Tris-HCl, pH 8.0, and 0.1 mM ethylene glycol bis(\(\beta\)-aminoethyl ether)-N\(^\cdot\)N\(^\cdot\)tetracetic acid). Samples containing 25 to 35 mg of protein in 5 ml were passed through a column of Sephadex G-200 (2.5 \(\times\) 45 cm) equilibrated with Buffer D. Protein was eluted in a sharp peak at the void volume followed by a broad shallow peak. The entire broad peak was collected, concentrated in a Diaflo filter apparatus, and dialyzed against Buffer C.

*Step 4*—The dialyzed material (10 to 40 mg) was passed through a column of DEAE-cellulose exactly as described in Step 2 above. Protein now separated into two peaks and only that material eluting in the second peak at a KCl concentration of 0.33 M KCl was collected, concentrated, and dialyzed against Buffer C.

*Step 5*—Dialyzed samples containing 10 mg of protein were applied to a column containing 7 to 10 g of hydroxyapatite (Bio-Gel HT1) equilibrated with 10 mM potassium phosphate, pH 7.0. The column was eluted with 400 ml of a linear gradient of potassium phosphate from 0.01 to 1 M. Samples of about 3.5 ml were collected and the purified protein making up the second peak was eluted in tubes 19 to 23. The protein was concentrated, dialyzed against Buffer C, and stored at -20\(^{\circ}\). The yield of purified protein is about 1 mg from 800 g of wet muscle tissue.

B. **Calsequestrin**

Calsequestrin was purified as described previously (12) except that the sequence of steps was altered. Column chromatography included sequential passage through DEAE-cellulose, hydroxylapatite, and Sephadex G-200, in that order.

C. **Low Molecular Weight Acidic Proteins**

Low molecular weight acidic proteins were eluted from DEAE-columns with calcequestrin (Step 2 of Section A) and from hydroxylapatite in a sharp peak immediately preceding calsequestrin. The pooled peak from hydroxylapatite was further fractionated into two classes, one containing predominantly proteins of molecular weights 33,000 and 38,000 and the other containing predominantly the 20,000 molecular weight species by passage of 10 mg of protein in 5 ml of Buffer D through a column of Sephadex G-200 (2.5 \(\times\) 45 cm) equilibrated with Buffer D. The broad peak of protein was divided into approximately equal portions and protein was concentrated and dialyzed against Buffer C and stored at -20\(^{\circ}\).

**Removal of Glycolytic Enzymes**

Phosphorylase and phosphorylase kinase were removed from sarcoplasmic reticulum by washing procedures. Sarcoplasmic reticulum (R1W) was stored overnight at 0\(^{\circ}\) at a protein concentration of 25 mg per ml in Buffer A. The solution was thawed and 1 ml was diluted with 1.5 ml of a solution of Buffer A. The suspension was centrifuged at 165,000 \(\times\) g for 25 min. The pellet was washed once more in Buffer A and stored at 10 mg per ml in Buffer A.

**Analytical Studies**

**Calcium Binding Studies**

Calcium binding was measured by dialysis equilibrium (12). In a standard assay, 0.4 mg of protein was dialyzed in a shaker bath for 40 hours at 4\(^{\circ}\) against 100 ml of a solution containing 500 \(\mu\)moles of Tris-HCl, pH 7.5, and 0.30 \(\mu\)Ci (4,000 cpm per ml) of \(^{45}\)CaCl\(_2\). Unlabeled CaCl\(_2\) and KCl were added as indicated. Samples (0.1 ml) from within and without the dialysis bag were dissolved in 10 ml of Aquasol (New England Nuclear) and were counted in a scintillation spectrometer. Protein was determined by the Lowry method (15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in 7.5\% acrylamide gel with 0.13\% bisacrylamide cross-linker (16). Samples, 200 \(\mu\)g, were boiled for 3 min in 0.1 M sodium dodecyl sulfate, 0.1\% 2-mercaptoethanol, and 50\% glycerol in 10 M sodium phosphate buffer, pH 7.0. They were then diluted with 0.1 ml of 0.1\% sodium dodecyl sulfate, 0.1\% 2-mercaptoethanol, and 50\% glycerol in 10 M sodium phosphate buffer. Samples, 20 \(\mu\)l, were applied on gels (0.5 \(\times\) 6 or 0.5 \(\times\) 12 cm) and electrophoresis was carried out at 8 ma per tube for 135 min or 240 min, respectively, with 0.1 M sodium phosphate, pH 7.0, in 0.1\% sodium dodecyl sulfate as electrode buffer. Gels were stained with Coomassie blue and destained electrophoretically.

Phosphorylase \(b\) activity was measured by the method of Cori et al. (17) and phosphorylase kinase activity was measured by the method of Wanson and Drochmanins (18).

**RESULTS**

We have previously reported that a deoxycholate extract of sarcoplasmic reticulum contains proteins which bind Ca\(^{2+}\) (12) and that chromatography of this extract on DEAE-cellulose,
hydroxyapatite, and Sephadex columns permits isolation of a series of five acidic proteins (12, 13). A sodium dodecyl sulfate-polyacrylamide gel electrophoretic profile of sarcoplasmic reticulum is presented in Fig. 1A. We have labeled the five acidic proteins as the high affinity calcium-binding proteins of molecular weight 55,000 ± 1,000, calsequestrin of molecular weight 46,500 ± 2,500, and low molecular weight acidic proteins of molecular weights 38,000, 33,000, and 20,000. We have found variations in molecular weight to be dependent on acrylamide concentration and length of gel column at a constant pH of 7.0. For calsequestrin the variation was 10%; for other proteins the variation was less.

The purification of calsequestrin has been documented (12). A second acidic protein, migrating in sodium dodecyl sulfate-polyacrylamide gels between the ATPase protein and calsequestrin has now been purified to homogeneity by procedures described under "Materials and Methods." Fig. 1B shows the purity of the protein and Fig. 1C confirms that it migrates with a protein prominent in disc gel electrophoretic profiles of sarcoplasmic reticulum.

Fig. 2A shows disc gel electrophoretic profiles of the series of low molecular weight acidic proteins which were separated from calsequestrin during hydroxylapatite chromatography (see "Materials and Methods"). This protein fraction was then passed through Sephadex G-200 to achieve a further separation based on molecular weight differences (Fig. 2, B and C). During disc gel electrophoresis these proteins migrated with the proteins designated low molecular weight acidic proteins in Fig. 1A. The arrow in Fig. 2B points to the protein of molecular weight 33,000 and the arrow in Fig. 2C points to the protein of molecular weight 20,000.

The molecular weights (9, 14, 19-22) and even the existence (14) of some of the acidic proteins are points disputed in the literature. Ikemoto et al. (14) have reported the isolation of a protein with essentially the identical properties of calsequestrin but they have reported the molecular weight to be 55,000 and, moreover, they have claimed that only two proteins exist in their preparations of fragmented sarcoplasmic reticulum. Fig. 3 is a disc gel electrophoretic profile of their preparation of fragmented sarcoplasmic reticulum. In this preparation there were two major proteins which we could identify with the ATPase and calsequestrin but, under the conditions of electrophoretic separation used in our laboratory, all of the acidic proteins labeled in Fig. 1 could also be seen. When the high affinity calcium-binding protein and calsequestrin were mixed with fragmented sarcoplasmic reticulum prior to disc gel electrophoresis, calsequestrin was found to migrate with one of the two major protein bands while the high affinity calcium-binding protein migrated with a less prominent protein band (Fig. 3). We regard this as excellent evidence that calsequestrin and the protein designated by Ikemoto et al. (14) as the "55,000-dalton protein" are identical in
molecular weights as well as in other properties. In an attempt to estimate whether the molecular weight of the protein was 55,000 or 44,000, we have added pyruvate kinase (mol wt 57,000) and aldolase (mol wt 40,000) to preparations of fragmented sarcoplasmic reticulum. Fig. 3 shows that the protein band identified as calsequestrin migrates well ahead of the 57,000 molecular weight marker and behind the 40,000 molecular weight marker. In fact, it runs with an Rf equivalent to a molecular weight of 46,500.

Removal of Glycolytic Proteins from Sarcoplasmic Reticulum—Our preparation of sarcoplasmic reticulum (23), like that of other laboratories (cf. Ref. 18), is contaminated with glycolytic enzymes, notably, phosphorylase and phosphorylase kinase. The possibility existed that one of the acidic proteins which we have isolated was the subunit of phosphorylase with high affinity for Ca2+ (24). We have ruled out this possibility on the basis of three observations: (a) phosphorylase kinase activity cannot be detected in any of the acidic protein fractions; (b) phosphorylase kinase activity (Sigma Chemical Co.) is eluted from DEAE-cellulose under conditions where the acidic proteins from sarcoplasmic reticulum are retained; and (c) phosphorylase and phosphorylase kinase activities can be washed away from sarcoplasmic reticulum (Table I) leaving the acidic proteins bound to the membrane. The acidic proteins can be visualized in undiminished quantity in the membranes by disc gel electrophoresis and can be isolated from membranes greatly depleted of glycolytic enzymes.

Ca2+ Binding by Acidic Proteins—Calcium binding was measured in 5 mM Tris-HCl, pH 7.5, in the presence or absence of 0.1 M KCl and the binding curves were analyzed using Scatchard plots. The use of KCl has permitted us to distinguish high, medium, and low affinity binding sites for Ca2+.

The protein of molecular weight 55,000 bound a large amount of Ca2+ when dialyzed against solutions containing Ca2+ in excess of 100 μM in the absence of KCl (Fig. 4). Maximum binding amounted to about 460 nmoles per mg of protein or about 25 moles per mole and the dissociation constant (Kd) for binding was about 120 μM. When Ca2+ binding was measured in the presence of 0.1 M KCl, the high capacity binding constant was shifted to a value greater than 5,000 μM but in the range of 1 to 10 μM Ca2+, significant Ca2+ binding was observed. In this low Ca2+ concentration range the protein bound 16 to 22 nmoles of Ca2+ per mg or about 1 mole of Ca2+ per mole of protein with a dissociation constant between 2 and 4 μM (Fig. 5).

Calsequestrin has previously been reported to bind 700 to 900 nmoles of Ca2+ per mg of protein with a medium affinity (12). Fig. 6 confirms this and also shows that the capacity of calsequestrin for Ca2+ binding is not diminished by the presence of 0.1 M KCl. The dissociation constant for the complex is, however, increased to about 800 μM from about 60 μM. In the presence of 0.1 M KCl there is clearly no high affinity Ca2+ binding by calsequestrin.

Figs. 7 and 8 show the Ca2+ binding capacity of the low molecular weight acidic protein fractions in the presence and ab-

![Image](http://www.jbc.org/)

**Table I**

<table>
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<th>Fraction</th>
<th>Phosphorylase Specific Activity (units/mg)</th>
<th>Phosphorylase Total Activity (units)</th>
<th>Phosphorylase Kinase Specific Activity (units/mg)</th>
<th>Phosphorylase Kinase Total Activity (units)</th>
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<td>Sarcoplasmic reticulum</td>
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<td>7.6</td>
<td>380</td>
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<tr>
<td>First supernatant</td>
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<td>45</td>
<td>0.9</td>
<td>45</td>
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<tr>
<td>First pellet</td>
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<td>20</td>
<td>0.3</td>
<td>13</td>
</tr>
<tr>
<td>Second supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second pellet</td>
<td></td>
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</tbody>
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![Image](http://www.jbc.org/)

**Fig. 5.** A Scatchard plot of Ca2+ binding by the high affinity Ca2+-binding protein.
the apparent high affinity Ca\(^{2+}\)-binding constant was increased 10-fold and the amount of Ca\(^{2+}\) bound was reduced to less than 1 mole per mole of protein. Low affinity Ca\(^{2+}\) binding could be observed even in the presence of KCl.

Table II summarizes constants for Ca\(^{2+}\) binding by the various proteins. In the presence of 0.1 M KCl, that is, under conditions approximating intracellular salt concentrations, calsequestrin displayed high capacity Ca\(^{2+}\) binding with medium affinity. All of the other proteins had high capacity Ca\(^{2+}\) binding with lower affinities. The protein of molecular weight 55,000 displayed a unique high affinity Ca\(^{2+}\)-binding site equivalent to 1 mole per mole of protein. The low molecular weight acidic proteins had apparent high affinity Ca\(^{2+}\)-binding sites in the absence of KCl but the dissociation constant was increased 10-fold in the presence of KCl and the concentration of sites was reduced to less than 1 mole per mole of protein.

**DISCUSSION**

When the sarcoplasmic reticulum is disrupted with deoxycholate in the presence of 1 M KCl, a series of acidic proteins is released in water-soluble form. The purified proteins have differing affinities for binding of Ca\(^{2+}\) (13); in this report we have investigated the specificity of Ca\(^{2+}\) binding in the presence of 0.1 M KCl and have found that this procedure provides further distinction among the various acidic proteins.

We previously reported that the low molecular weight acidic protein fractions had high affinity Ca\(^{2+}\)-binding capacity in the absence of KCl (13). They also bound a limited amount of Ca\(^{2+}\) with apparent high affinity as previously reported (13). The proteins bound 20 to 30 nmoles per mg of protein even when the Ca\(^{2+}\) concentration was reduced to 1 \(\mu\)M. In the presence of 0.1 M KCl, however,

<table>
<thead>
<tr>
<th>Protein</th>
<th>+ 0.1 M KCl</th>
<th>- KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_d)</td>
<td>(n)</td>
</tr>
<tr>
<td>High affinity</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Ca(^{2+})-binding protein</td>
<td>5000</td>
<td>400</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>800</td>
<td>850</td>
</tr>
<tr>
<td>20,000 (mol wt)</td>
<td>1400</td>
<td>1000</td>
</tr>
<tr>
<td>33,000-38,000 (mol wt)</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>20,000 (mol wt)</td>
<td>780</td>
<td>580</td>
</tr>
</tbody>
</table>

We had earlier speculated that these proteins might be located on the exterior of the membrane, providing high affinity Ca\(^{2+}\)-binding sites. We also suggested that they might correspond to the surface particles seen in electron micrographs. These hypotheses have proven untenable on two grounds. First, the apparent high affinity Ca\(^{2+}\)-binding sites of these proteins are abolished in isotonic KCl. Second, we have shown in a companion paper (25) that the surface coat is a structural feature of the ATPase enzyme and consequently could not be composed of acidic proteins. The role of the low molecular weight acidic proteins in the Ca\(^{2+}\) transport system of sarcoplasmic reticulum and their localization are, therefore, still open questions.
Calsequestrin has been found to retain its high capacity Ca\(^{2+}\) binding activity in the presence of 0.1 m KCl. The protein still bound 800 to 900 nmoles of Ca\(^{2+}\) per mg of protein but the dissociation constant was shifted from about 60 to about 800 \(\mu\)M when KCl was added to the assay medium.

Ikemoto et al. (14) have reported the isolation of a protein whose characteristics indicate that it is calsequestrin. They have pointed out some apparent physical differences and, in addition, have shown that the protein binds 700 to 800 nmoles of Ca\(^{2+}\) per mg in the presence of 0.1 m KCl and that it undergoes a change in its circular dichroism spectrum at 222 nm in response to the presence of Ca\(^{2+}\). Our experiments support the view that the protein isolated by Ikemoto et al. (14) is in fact calsequestrin. The difference in molecular weight is a difference in measurement only, since calsequestrin migrates together with another protein of 55,000 molecular weight. DEAE-cellulose fractionation was pure. All of the acidic proteins described here precipitate with Ca\(^{2+}\) and, moreover, calsequestrin is bound to the membrane in essentially the same fashion (13, 25, 27) and both appear to be localized on interior sites.

We have reported slight stimulation of transport upon the addition of the high affinity calcium-binding protein vesicles (13). The high affinity binding protein of molecular weight 55,000 is clearly not the “55,000-dalton protein” described by Ikemoto et al. (14). The high affinity Ca\(^{2+}\)-binding protein is, however, present in fragmented sarcoplasmic reticulum prepared by Ikemoto et al. (14) (Fig. 3).

The role of the high affinity Ca\(^{2+}\)-binding protein, like that of any of the acidic proteins, is still unknown. This protein and calsequestrin are bound to the membrane in essentially the same fashion (13, 25, 27) and both appear to be localized on interior sites.

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**REFERENCES**

3. MARTONOSI, A. (1971) Bioembranes 1, 191
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