Finally one can make profitable use of the fact that the ionic radii of Ca^{2+} and Cd^{2+} are very similar and substitute the spin I = ½ nucleus ^{113}Cd for Ca^{2+}. Comparative ^{1}H NMR studies of a number of calcium binding proteins indicate that the overall tertiary structures of the Ca^{2+} and Cd^{2+} loaded form of the proteins are very similar. NMR spectra of ^{113}Cd bound to proteins — in many cases showing one or more fairly narrow signal(s) — give valuable complementary information on such phenomena as cooperativity of ion binding, the nature of the protein ligands and structural changes of the protein.

Some recent applications concern regulatory calcium binding proteins like skeletal and heart muscle troponin-C and calmodulin as well as enzymes and proenzymes like trypsinogen and trypsin, prophospholipase A_2 and phospholipase A_2. Ca^{2+} exchange rates determined from ^{43}Ca NMR indicate the on-rates to be about 10^8 sec^{-1} M^{-1} for the regulatory proteins but about two orders of magnitude lower, or 10^6 sec^{-1} M^{-1}, for the proenzyme/enzymes.

Through ^{113}Cd NMR studies of native calmodulin and different proteolytic fragments the sequence of binding of Cd^{2+} and Ca^{2+} to the four calcium binding sites has been established.

^{113}Cd NMR has been used to characterize the structural changes associated with the transformation of trypsinogen to trypsin or as a result of the formation of binary or ternary complexes. The ^{113}Cd NMR data indicate that the structure of the calcium binding site remains essentially unchanged.

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C3

Studies on the Regulation of Muscle Contraction by Troponin

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Two major areas of our current research interests will be discussed. The first deals with our attempts to understand the Ca^{2+}-binding properties of Tn *in situ* and the second involves our recent antibody studies which are aimed at elucidating the structure of the Tn complex as well as the function of the various subunits in Ca^{2+} regulation.

Two different methods, one direct and one indirect, have been used to measure Ca^{2+} binding to troponin in thin filaments. Both methods demonstrate that actin lowers the affinity of the Ca^{2+} specific sites on Tn for Ca^{2+} by approximately an order of magnitude while the affinity of the Ca^{2+}–Mg^{2+} sites remains unchanged. Furthermore, the Ca^{2+} dependence of myofibrillar ATPase activation corresponded exactly with Ca^{2+} binding to the Ca^{2+}-specific sites as measured by the fluorescence change of TnC_{DANZ} specifically incorporated into myofibrils (H. G. Zot and J. D. Potter, *J. Biol. Chem.*, *257*, 7678 (1982)). These results lead us to two major conclusions. The first is that if actin alters the structure of Tn (as indicated by a change in Ca^{2+} affinity of the Ca^{2+}-specific sites) then Ca^{2+} binding to the Ca^{2+}-specific sites must alter the structure of actin. Since previous studies have shown that the Ca^{2+}-specific sites are the sites involved in regulating actomyosin interaction, this postulated change in actin structure brought about by Ca^{2+} binding to these sites, may be the key event in muscle activation. The second conclusion is that the exchange of Ca^{2+} with the Ca^{2+}-specific sites in muscle is probably faster than previously estimated from studies on isolated Tn where the affinity of the Ca^{2+}-specific sites is an order of magnitude higher. This is important since these sites must exchange Ca^{2+} rapidly in order to be able to respond to the rapid $[Ca^{2+}]$ transients which occur in muscle.

A monoclonal antibody (IgG-$\kappa_2\gamma_2$) to STnI has been produced using the somatic cell fusion of spleen cells from CB_6F_1/J mice immunized with STnI with cells of the myeloma cell line SP2/O-Ag-14. The reaction of this antibody (C135) towards STnI has been determined using a) the ELISA technique, b) a triple sandwich immunodot technique, and c) by immunoblot on nitrocellulose after gel electrophoresis of skeletal myofibrils. C135 also interacts with cardiac TnI (CTnI) suggesting that both STnI and CTnI share

a common antigenic determinant. The effect of C135 on the Ca^{2+} dependence of the myofibrillar ATPase activity was also investigated. At low Ca^{2+} concentrations, where the ATPase was inhibited, the antibody had no effect. However, in the presence of Ca^{2+}, where the myofibrils were fully activated, the antibody greatly inhibited the ATPase activity. These results suggest that the binding of the antibody to STnI in the myofibrils 'locks' the conformation of this troponin subunit in an inhibitory state, similar to the one in which STnI is still bound to actin in the absence of Ca^{2+}. Work is in progress to determine the binding site of C135 on fragments of STnI generated by CNBr cleavage.

These two areas will be discussed in terms of our current overall view of the mechanism of Ca^{2+} regulation of muscle contraction.

C4

Calcium Involvement in the Protein-Membrane Systems of Blood Coagulation

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Blood coagulation is an extracellular process that occurs in a medium containing millimolar concentrations of calcium and utilizes this ion in several ways. One major utilization is by the vitamin K-dependent, γ-carboxyglutamate-containing proteins. Four of these proteins are known to function as enzymes and/or substrates for membrane-bound enzyme-catalyzed reactions of the blood-clotting cascade. Three additional vitamin K-dependent proteins are known in the plasma, one of which probably acts as an enzyme in a membrane requiring reaction that decreases coagulation. All of these plasma proteins have homologous amino terminal sequences of about 40 residues which contain 10 to 12 γ-carboxyglutamic acid residues.

Calcium functions to aid binding of these vitamin K-dependent proteins to membranes containing acidic phospholipids. Acidic phospholipids are found primarily in cytosolic membranes and are nearly absent on the external surface of the plasma membranes. Exposure of cytosolic membranes through cell damage may therefore constitute an important signal enabling blood coagulation to occur. Calcium binding to γ-carboxyglutamic acid-containing plasma proteins causes a protein conformational change which is prerequisite to protein–membrane binding. In the case of prothrombin, the protein conformational change apparently involves a trans to *cis* proline isomerization (Pro_{22}) which has a characteristic reaction half-time of 90 minutes at 0 °C and an activation energy of 21 kcal/mol. The conformational changes for all of these proteins result in changes in intrinsic protein fluorescence as well as other spectral properties and are cooperative with respect to calcium. Very little selectivity for multivalent metal ions is displayed by the proteins and the sites have an average K_D of 10^{-4} to 10^{-3} *M* calcium which varies slightly for the different proteins. Prothrombin, the most thoroughly studied protein, binds 6 calcium ions in parallel with the protein conformation change with an average K_D of 0.25 m*M* calcium. Many other ions show site specificity and three bound ions (Mn, Cd, Lanthanides) are sufficient to cause the conformational change.

Calcium serves a second role in these protein–membrane interactions which appears to involve the acidic phospholipid residues. This second calcium-dependent process is also cooperative with respect to metal ion and shows a similar affinity for calcium. Approximately four calcium ions and eight acidic phospholipid residues are required for this second process. Metal ion specificity is observed in this step with calcium and strontium functioning fully while magnesium and manganese fail to function at all.

The precise structure of the protein–membrane interface is not known but studies with phospholipid bilayers and monolayers indicate involvement of the phospholipid headgroup only. The favored model is calcium bridging between ligands of the protein and the phospholipid. Rapid kinetic studies indicate that the metal ions responsible for maintaining protein conformation are in rapid exchange with solvent ions even in the membrane-bound state.

C5

Binding and Transport of Calcium by Sarcoplasmic Reticulum ATPase

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Sarcoplasmic Reticulum (SR) membrane can be isolated from striated muscle in the form of sealed vesicles containing a high density of ATPase protein that accounts for approximately half the membrane mass. The ATPase polypeptide units are composed of polar segments protruding from the outer surface of the membrane into the aqueous medium, and hydrophobic segments intruding the membrane bilayer. The specific function of the SR ATPase is to take up Ca^{2+} from the medium *outside* the vesicles, and release it into the aqueous medium *inside* the vesicles. Thereby a transmembrane Ca^{2+} gradient is formed, deriving free energy from ATP hydrolysis.