# Minisymposium: Calcium Binding Sites in Proteins

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## C1

### Calcium in Biological Systems

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In physiological fluids calcium ion takes part in many processes. Among these are muscle contraction, microtubule formation, hormonal responses, exocytosis, fertilization, neurotransmitter release, blood clotting, protein stabilization, intercellular communication, mineralization, and cell fusion, adhesion, and growth. Most of these $Ca^{2+}$ related activities occur by interactions with proteins, which $Ca^{2+}$ may stabilize, activate, and modulate.

In extracellular fluids the free or weakly bound $Ca^{2+}$ concentration is about 1 *mM*. Within many cells the free $Ca^{2+}$ concentration in the cytosol is only 0.1 $\mu M$, $10^{-4}$ times less than in extracellular fluids. Cell membranes contain pumps, Ca-ATPases, that aid in maintaining the extraordinary concentration gradient. However, a substantial amount of $Ca^{2+}$ occurs within cells, some of it bound tightly to proteins. In response to a stimulus the free $Ca^{2+}$ concentration may increase about 10 times. Thus proteins that participate in these responses possess $Ca^{2+}$ dissociation constants in the $\mu M$ range. The cytosolic $Ca^{2+}$ concentration change is achieved rapidly, and free $Ca^{2+}$ serves as a messenger or trigger for other interactions.

$Ca^{2+}$ sites in proteins are composed of negatively charged and neutral oxygen donors; nitrogen donors seem unlikely, and none have been found. Protein oxygen donors derive from carboxylate groups, carbonyl oxygens of the amide backbone, and hydroxy groups of serine and threonine side chains.

$Ca^{2+}$ varies in its coordination number and bond lengths. The frequency of $Ca^{2+}$ coordination numbers decreases in the order $8 > 7 > 6 > 9$. Coordination about $Ca^{2+}$ is basically ionic and spherical. $Ca^{2+}$–O bond distances range from 2.3 to 2.6 Å. In solution, even within a single complex, there may be variability in bond distances and, in many cases, coordination number. To bind $Ca^{2+}$, proteins provide a pocket of appropriate size and shape with two or more negatively charged carboxylate side chains. Specific applications of these general principles appear in other papers in this symposium and in Vol. 17 of *'Metal Ions in Biological Systems'*, H. Sigel, ed.

Except for the charge difference, usually not crucial, tripositive lanthanide ions mimic many $Ca^{2+}$ properties. Energy transfer from a nearby excited aromatic chromophore produces $Tb^{3+}$ luminescence. The spectrum and relative intensity compared to the total luminescence intensity of the circularly polarized luminescence from parvalbumin and troponin-C are nearly identical. Specific $Ca^{2+}$ binding sites in the two kinds of proteins are therefore similar. The excitation spectrum identifies the donor group in the energy transfer process as a phenylalanine side chain in parvalbumin and a tyrosine side chain in troponin-C. The two amine acids comprise homologous pairs in the two proteins.

## C2

### $^{25}Mg$, $^{43}Ca$ and $^{113}Cd$ NMR Studies of Calcium Binding Proteins

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NMR spectroscopy offers many alternative ways of studying the properties of calcium and magnesium binding proteins. One obvious way is to observe the spectra of spin I = ½ nuclei like $^{1}H$, $^{13}C$ and $^{15}N$ under various conditions as regards ion concentration, pH, temperature *etc.* Information pertaining to structure and dynamics of the protein may be gained in this way. As a result of recent developments in methodology and instrumentation NMR of the quadrupolar ions $^{25}Mg$ and $^{43}Ca$ has also developed into a useful tool in the study of calciproteins (*cf.* ref. 1–5). Through the combined use of isotopically enriched $^{25}Mg$ and $^{43}Ca$, FT NMR techniques, high magnetic fields and a solenoid type of probe design, NMR studies of these cations are now feasible at millimolar, or even submillimolar, concentrations. The general type of information that can be obtained from $^{25}Mg$ and $^{43}Ca$ NMR is:

*(i)* association constants in the range $1–10^{4}\ M^{-1}$;

*(ii)* the competition of other cations for the $Ca^{2+}$ and/or $Mg^{2+}$ binding site(s);

*(iii)* the effects of other protein ligands (drugs, *etc.*) on the ion binding;

*(iv)* the apparent pK values of the groups involved in $Ca^{2+}$ and $Mg^{2+}$ binding;

*(v)* dynamic parameters *i.e.* chemical exchange rates and activation parameters or correlation time(s) characterizing the ion binding site(s).

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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3 S. Forsén, T. Andersson, T. Drakenberg, E. Thulin and M. Swärd, 'Feder. Proc.', Vol. *41*, no. 13 (November issue) (1982).
4 K. J. Neurohr, T. Drakenberg and S. Forsén, 'Magnesium-25 and Calcium-43' in "NMR of Quadrupolar and Less Receptive Nuclei: Chemical and Biological Applications" (P. Laszlo, ed.). Acad. Press, New York, (1983), in press.
5 H. Vogel, T. Drakenberg and S. Forsén, 'Calcium-Binding Proteins' in "NMR of Quadrupolar and Less Receptive Nuclei: Chemical and Biological Applications", (P. Laszlo, ed.). Acad. Press, New York, (1983), in press.

## 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