3.13: Paravalbumin and Calbindins D 9K D9K and D 28K D28K

A few intracellular Ca^{2+}-binding proteins have been discovered that by sequence homology clearly belong to the CaM-TnC family with Ca^{2+} sites of the "EF-hand"-type, but that do not appear to exert a direct regulatory function. Parvalbumins ($M_r \approx 12$ kDa), calbindin D_{9K} ($M_r \approx 8.7$ kDa) and calbindin D_{28K} ($M_r \approx 28$ kDa) belong to this group. *Parvalbumin(s)* exist in two main types, α and β, found in large quantities in the white muscle of fish, amphibia, and reptiles, but also in different mammalian tissues,[116,117] including neurons of the central and peripheral nervous system. The molecule has two fairly strong Ca^{2+}- binding sites (see Table 3.2). The x-ray structure of carp parvalbumin was solved in 1973 by Kretsinger *et al.*,[118] and for a decade provided the basis for all discussions on intracellular Ca^{2+}-binding proteins. The concept of the canonical "EF-hand" Ca^{2+}-binding site originated from the parvalbumin work, and the name "EF" derives from the labeling of the two helices that flank the second of the two Ca^{2+} sites in parvalbumin, as shown in Figure 3.23.

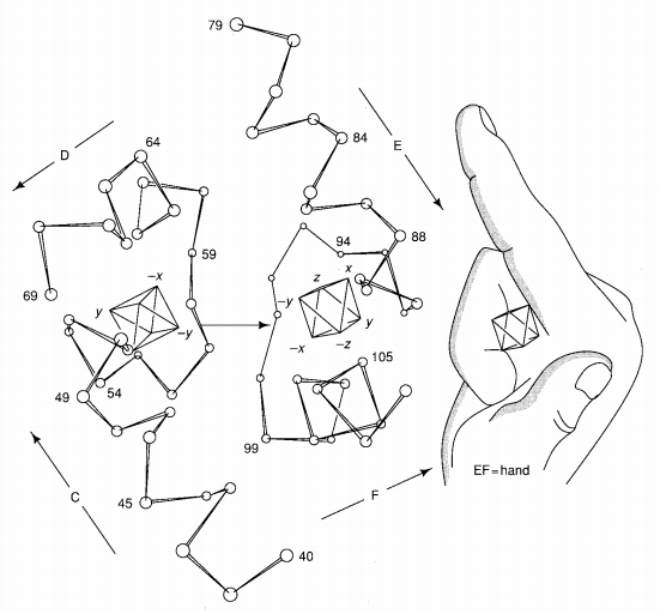

Figure 3.23 - Structure of the Ca^{2+}-binding sites of carp parvalbumin. The Ca^{2+} ions are depicted as regular octahedra making six ligand contacts with oxygen atoms at each vertex, labeled x, y, z, -x, -y, -z. The helix-loop-helix structure that forms a Ca^{2+}-binding site can be regarded as a hand with the forefinger representing one helix (e.g., the E-helix) in the plane of the figure, the thumb oriented perpendicular to the plane representing the second helix (the F-helix), and the remaining fingers make up the Ca^{2+}-binding loop. After Kretsinger and Barry.[118]

If the first Ca^{2+} ligand in the approximately octahedral coordination sphere is given number 1 (or "x") the others come in the order 3("y"), 5("z"), 7("-y"), 9("-x"), and 12("-z"). In the second site of parvalbumin, "-x" is actually a H_2O molecule, but in the first site it is the carboxylate of a Glu. Studies[118] of putative Ca^{2+}-binding sites in other proteins with known primary sequences led to the generalized EF-hand structure—including residues in the flanking α-helices—shown in Figure 3.24.

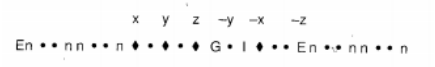

Figure 3.24 - One consensus EF-hand sequence including residues in the flanking α-helices; x, y, z, -x, -y, - z denote positions in the octahedral Ca^{2+} coordination sphere. E—glutamic acid residue, G—glycine residues, I—isoleucine residue, n—nonpolar residue, ♦—a residue with a nonaromatic oxygen-containing side chain (i.e., Glu, Gin, Asp, Asn, Ser, or Thr), and •—nonspecific residue.

This sequence, with minor modifications, has been widely used in searching for "EF-hands" in libraries of amino-acid (or DNA) sequences of new proteins with unknown properties. In this way, *calbindin* D_{28k} a protein with unknown function, initially discovered in chicken intestine, but later found also in brain, testes, and other tissue, has been shown to have four EF-hand sites.[119]

Recently two structures of carp parvalbumin, both with a resolution of 1.6 Å, were published.[120] One of these structures is the native calcium-loaded form of the protein; the second is the structure of parvalbumin in which Ca^{2+} has been replaced by Cd^{2+}. No significant differences are observed upon replacement of calcium by cadmium. ^{113}Cd has a nuclear spin of $I = \frac{1}{2}$, making it much

more amenable to NMR studies than the quadrupolar ^{43}Ca ($I = \frac{7}{2}$), This study supports the use of ^{113}Cd NMR as a tool for the study of calcium-binding proteins.[121]

The function of parvalbumin has long been assumed to be that of buffering Ca^{2+} in muscle cells, i.e., taking up Ca^{2+} ions released from Ca^{2+}-troponin complexes, thereby ensuring that the cytoplasmic levels of free Ca^{2+} are always kept very low, even during short bursts of muscle activity,[122] The widespread occurrence of parvalbumin in non-muscle tissue indicates that it probably has other roles as well.

Calbindin D_{9k} ($M_r \approx 8.7$ kDa) is another intracellular Ca^{2+}-binding protein with unknown function. It was briefly mentioned in connection with Ca^{2+} uptake and transport in the intestine and placenta (Section IV.A). Like the avian calbindin D_{28k} the D_{9k} calbindin has been observed in many types of tissue. The homology between the D_{9k} and D_{28k} calbindins is much less than the name suggests; both their syntheses are, however, regulated by vitamin D. The x-ray structure of bovine calbindin D_{9k} has been determined[123] and refined to a resolution of 2.3 Å, and a three-dimensional *solution* structure of porcine calbindin D_{9k} is also available.[124] The average solution structure calculated from NMR data is shown in Figure 3.25 (See color plate section, page C-10.)

The protein has four main α-helices and two Ca^{2+}-binding loops (I and II). The interior of the molecule shows a loose clustering of several hydrophobic side chains; in particular, three phenylalanine rings come very close in space. The Ca^{2+}-binding loops constitute the least-mobile parts of the molecule. The crystallographic temperature factors have pronounced minima in these regions, with the lowest overall B-factor observed in loop II. Both Ca^{2+} ions are roughly octahedrally coordinated with protein oxygen atoms. There are some striking differences between the two sites, however. Whereas the C-terminal site (II) has a general structure very similar to the archetypal "EF-hand," as observed in CaM, sTnC, and parvalbumin, the N-terminal site (I) has an extra amino-acid residue inserted between vertices x and y, and z and -y (see Figure 3.24). As a consequence, the peptide fold in site I is different from that in site II. Three carboxylate groups are ligands in site II, but in site I there is only one.

Despite this marked difference in charge and peptide fold, the Ca^{2+} affinity of both Ca^{2+} sites is remarkably similar, as has been shown in a study in which site-directed mutagenesis was combined with different biophysical measurements.[37] Cooperative Ca^{2+} binding in the native calbindin D_{9k} (the "wild type") was first demonstrated at low ionic strength by means of the values of the two stoichiometric Ca^{2+}-binding constants, K_1 and K_2, which could be measured with good accuracy ($K_1 = 4.4 \times 10^8\ M^{-1}$ and $K_2 = 7.4 \times 10^8\ M^{-1}$). The effects of amino-acid substitutions in Ca^{2+} site I were primarily localized to this site, with virtually no effects on the structure or other biophysical properties pertinent to site II. The appearance of sequential Ca^{2+} binding in some of the calbindin mutants did allow the identification of ^1H NMR resonances that respond primarily to binding of Ca^{2+} to either one of the sites. This result in tum permitted an estimate of the ratio between the site-binding constants (K_A and K_B) in the wild-type protein and in one of the mutant proteins (Tyr-13 → Phe). In this way the reseachers[125] could assess, to within narrow limits, the free energy of interaction, $\Delta\Delta G$, between the two Ca^{2+} sites as 7.7 kJ/mol at low ionic strength and 4.6 kJ/mol in the presence of 0.15 M KCl. How this site-site interaction is transmitted on a molecular level is still unknown.

Through a combination of site-specific mutations and biophysical measurements, it has recently been demonstrated that carboxylate groups at the surface of the protein, but not directly ligated to the bound Ca^{2+} ions, have a profound effect on the Ca^{2+} affinity.[126] Neutralization of the surface charges reduces affinity and increases the stability of the protein toward unfolding by urea.[127]

A surprising discovery about the structure of bovine calbindin D_{9k} in solution has also been made recently.[128] Detailed analysis of the 2D ^1H NMR spectrum of wild-type calbindin has revealed that it exists as a 3:1 equilibrium mixture of two forms, corresponding to a *trans* and *cis* conformation around the Gly-42-Pro-43 peptide bond. The global fold appears essentially the same in the two forms, and structural differences are primarily located in the inter-domain loop in which Pro-43 is located.