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EDITED BY

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Calcium-Binding Proteins and the Molecular Basis of Calcium Action

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I. Introduction

Calcium is required for the maintenance of optimal growth and functioning of most living organisms. For example, calcium appears to be involved in the mechanism of action of many hormones, drugs, and toxins, regulation of secretion and contraction, digestion of food and absorption of nutrients, formation and maintenance of bone and teeth, regulation of blood clotting and wound healing, functioning of various enzymes and receptors, and regulation of photochemical events in plants and animals. While the molecular mechanisms by which calcium

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mediates these processes probably involves interactions with a variety of molecules, a large body of evidence has demonstrated the direct involvement of proteins in the molecular mechanisms of calcium action. Further, the recent elucidations of the types of protein structures used in several different mechanisms of calcium/protein interactions have allowed some correlation of calcium-binding protein structures with calcium-dependent activity.

In this review we discuss selected precedents that demonstrate some of the ways in which calcium is bound to a protein and how calcium is involved in protein function. The proteins used as examples are, in general, ones which appear to have a physiological requirement for calcium and ones for which extensive molecular and atomic detail is available (e.g., X-ray crystal structures and amino acid sequences). Four classes of calcium-binding proteins that reflect different uses of calcium in protein function and different ways in which proteins bind calcium ions are reviewed. These four classes are (1) proteins containing γ-carboxyglutamic acid, (2) calcium-binding lectins with emphasis on concanavalin A, (3) calcium-binding hydrolytic enzymes with emphasis on phospholipase A_2, and (4) calcium-modulated proteins with emphasis on a relatively ubiquitous protein (calmodulin), a tissue-specific protein (S100), and a hormone-induced protein (vitamin D-dependent calcium-binding protein).

Proteins which contain γ-carboxyglutamic acid are discussed as an example of proteins which bind calcium mainly by bidentate chelation. It is possible that these and other proteins which bind calcium with dissociation constants in the millimolar range, such as the calcium-binding phosphoproteins, may be a class of calcium-binding proteins that exert their calcium-dependent activities extracellularly. The most extensively characterized proteins that contain γ-carboxyglutamic acid are the proteins involved in the regulation of blood clotting. The main function of calcium in these proteins appears to be the promotion of protein interaction with extracellular supramolecular structures or surfaces. Variations of this calcium-binding theme may be used by other proteins when interface reactions are the main function of calcium.

The role of calcium in the calcium-binding lectin concanavalin A appears to be protein stabilization. In contrast to the simple chelation type of binding found in the γ-carboxyglutamic acid-containing proteins, multiple protein ligands are used to bind calcium in lectins. The protein ligands are mostly the oxygens from amino acid side chains, but one oxygen is contributed by a carbonyl group from the peptide backbone. The polyhedron that is formed by wrapping the peptide chain around the calcium has a common edge with an adjoining polyhedron that forms a transition metal-binding site. In thermolysin, a calcium-binding enzyme, there is a pair of calcium-binding sites reminiscent of the calcium:manganese paired-ion sites in concanavalin A. The paired calcium sites in thermolysin appear to enhance the thermal stability of the enzyme. Paired cation binding sites

may be a structure used by a protein when calcium is functioning to "lock" a particular conformation that enhances protein stability or activity.

Phospholipase A_2 and other hydrolytic enzymes are discussed as examples of the involvement of calcium in substrate binding and enzymic catalysis. The coordination of calcium at the active site of phospholipase A_2 is by six ligands that form an octahedron. In contrast to concanavalin A and the calcium-modulated proteins, phospholipase A_2 uses mostly oxygens from peptide carbonyl groups as protein ligands for the active site calcium. At least one side chain oxygen, the carboxylate oxygen of an aspartic acid residue, is also used. Proposed mechanisms for the role of the active site calcium in enzyme function include stabilization of a tetrahedral intermediate generated during conversion of substrate to product. Lower affinity (K_D = 20 mM) calcium-binding sites are also found in phospholipase A_2. These latter sites are away from the active site and are important in the conversion of zymogen to enzyme. The lower affinity calcium-binding sites are thought to be in the NH_2-terminal portion of the enzyme, a region that is involved in the interaction between the protein and micellar substrates. Such lower affinity calcium-binding sites that appear to stimulate interface reactions remind one of the role of calcium in promoting the interaction of γ-carboxyglutamic acid-containing proteins with membranes and phospholipids. Thus, phospholipase A_2 provides a precedent for a single protein having multiple uses of calcium and possible multiple calcium-binding structures, as well as being a well-characterized system in which calcium:protein:lipid interactions are involved in enzyme function.

Parvalbumin, calmodulin, vitamin D-dependent calcium-binding protein, and brain S100 are discussed as selected examples of calcium-modulated proteins. Calcium-modulated proteins reversibly bind calcium with dissociation constants in the nanomolar to micromolar range under physiological conditions. The tertiary structure and activity of these proteins are thought to be modulated by the reversible formation and dissociation of a calcium–protein complex. Because most of these proteins are intracellular and have dissociation constants that span the range of intracellular free calcium concentrations, they are postulated to be the major signal transducers of biological calcium signals. Two types of calcium-binding structures, both of which utilize an α-helix:loop:α-helix arrangement of the peptide chain, have been found in this class of proteins. In both of these structures the calcium-binding residues are located in the loop connecting the two α-helices.

One type of calcium-binding structure found in calcium-modulated proteins is referred to as an EF hand structure. This name is derived from the calcium-binding structure in parvalbumin that is formed by the E-helix, the F-helix, and the peptide loop connecting these two α-helices. The other type of calcium-binding structure has been found recently in one of the mammalian vitamin D-

dependent calcium-binding proteins. This latter structure is similar in general features to the EF hand structure. Both structures are octahedra, but the EF hand structure uses mostly oxygens from amino acid side chains as ligands whereas the vitamin D-dependent protein structure utilizes mostly carbonyl oxygens from the peptide backbone. As a result, the spacing of calcium ligands in the amino acid sequence of the vitamin D-dependent protein is different from that found in an EF hand type of structure. Interestingly, the vitamin D-dependent protein has a second calcium-binding site that utilizes an EF hand structure. One proposal for the role of these two different types of structures in the same protein is that the EF hand structure might be involved in cellular responses to calcium signals while the other site might be occupied by calcium in the resting cell and would, therefore, be mainly involved in the stabilization of protein structure or conformation. The amino acid sequences of two S100 polypeptides have regions that are homologous with the vitamin D-dependent protein and might also have both of these calcium-binding structures. Calmodulin has four regions of amino acid sequence that appear to only have the sequence requirements for formation of an EF hand structure. Thus, all calcium-modulated proteins examined to date appear to have amino acid sequences that are capable of forming EF hand types of structures but may, in addition, have other calcium-binding structures.

The division of calcium-binding proteins into classes based upon limited structural and functional information is a subjective decision. In fact, the entire process of selecting material and writing a review is a highly personal endeavor. A number of calcium-binding proteins are not discussed in this article, and entire articles have dealt with some proteins which are only briefly mentioned here. We hope that by a brief discussion of selected types of calcium-binding proteins we might provide the interested biologist with a limited insight into the complexities as well as the generalities that are emerging in our knowledge of calcium-binding proteins and the molecular basis of calcium action in cellular processes.

II. Proteins Containing γ-Carboxyglutamic Acid

Extensive investigations have been done on a class of calcium-binding proteins containing the novel amino acid 3-amino-1,1,3-propane-tricarboxylic acid, commonly called 4-carboxyglutamic or γ-carboxyglutamic acid (Gla). Gla occurs as a vitamin K-dependent, posttranslational modification of glutamic acid residues. This modified amino acid is present in at least seven plasma proteins and several tissue-associated proteins. The presence of Gla in the plasma proteins apparently confers a number of physical and functional properties including the ability to bind calcium with a dissociation constant in the millimolar range and the ability to bind phospholipid in a calcium-dependent manner. The function of Gla in tissue-associated proteins is less clear but may be related to their ability to

bind calcium salts as suggested by their presence in extracellular calcium deposits.

Prothrombin is the prototypical Gla-containing plasma protein, especially with regard to structural characteristics and calcium- and phospholipid-binding properties. Likewise, BGP (or bone Gla-containing protein) has been more extensively investigated than other tissue-associated proteins containing Gla. The discussion which follows will, therefore, concentrate on these proteins as examplary of their respective subclass of Gla-containing proteins.

A. Background

The discovery of Gla was a result of Henrick Dam's observation (1935a,b) that vitamin K-deficient chickens displayed bleeding abnormalities due to a decrease in the amount of functional prothrombin in their plasma. Reduced levels of the clotting factors prothrombin, Factor VII, Factor IX, and Factor X were also reported in humans and cows following treatment with vitamin K antagonists such as dicumerol (Owens *et al.*, 1951; Aggeler *et al.*, 1952; Briggs *et al.*, 1952; Hougie *et al.*, 1957; Stenflo, 1970). An abnormal form of prothrombin was postulated by Hemker *et al.* (1963) and later shown by Stenflo (1970) to be present in plasma of the dicumerol-treated animals. The lack of activity of the abnormal prothrombin was then demonstrated (Nelsestuen and Suttie, 1972; Stenflo and Ganrot, 1972) to be due to its inability to bind calcium and phospholipid. Subsequently, the vitamin K-dependent structure responsible for these activities was shown (Magnusson *et al.*, 1974; Nelsestuen *et al.*, 1974; Stenflo *et al.*, 1974) to be γ-carboxyglutamic acid. More extensive accounts of the history of vitamin K and vitamin K-dependent proteins can be found in several reviews (Almquist, 1975; Stenflo, 1976; Suttie, 1977, 1978, 1980a,b).

The demonstration of γ-carboxyglutamic acid in proteins was hampered for years because under conditions of acid hydrolysis used in amino acid analysis Gla is quantitatively decarboxylated to glutamic acid (Hauschka *et al.*, 1975; Hauschka, 1977). Gla was originally identified (Stenflo *et al.*, 1974) by proton nuclear magnetic resonance spectroscopy and mass spectrometry. Release of stable Gla from peptide linkage can be achieved by alkaline hydrolysis (Hauschka *et al.*, 1975; Hauschka, 1977). The amino acid can then be quantitatively determined using an amino acid analyzer (Hauschka, 1977; Tabor and Tabor, 1977). A number of compounds elute from analyzer columns close to Gla, so the identification of the presumptive Gla peak should be confirmed by its disappearance upon acid hydrolysis (Hauschka, 1977). Several other chemical and chromatographic methods have been described for the detection and quantitation of Gla (Howard and Nelsestuen, 1974, 1975; Zytkovicz and Nelsestuen, 1975; Fernlund and Stenflo, 1980b; Grundberg *et al.*, 1980; Low *et al.*, 1980; Petersen *et al.*, 1980), but definitive identification of Gla is probably best accom-

plished by mass spectrometry (Magnusson *et al.*, 1974; Morris *et al.*, 1976; Carr *et al.*, 1981).

B. Synthesis of γ-Carboxyglutamic Acid

The synthesis of γ-carboxyglutamic acid and the metabolism of vitamin K have been described in several reviews and the proceedings of a recent symposium (Suttie and Jackson, 1977; Bell, 1978; Stenflo, 1978; Olson and Suttie, 1978; Suttie, 1978, 1980a,b,c; Suttie *et al.*, 1980b) and are only summarized in this article.

Three forms of vitamin K enter the liver vitamin K cycle: the quinone, the hydroquinone, and the 2,3-epoxide. The quinone and hydroquinone can be interconverted by a microsomal bound NAD(P)H-linked reductase. Vitamin KH_2 (the hydroquinone form) can be converted to the 2,3-epoxide form by a microsomal internal monooxygenase. The epoxide is in turn reduced to the quinone by a warfarin-sensitive enzyme, 2,3-epoxide reductase. The reduced vitamin K is an essential cofactor for the vitamin K-dependent carboxylase which carboxylates protein-bound glutamyl residues to γ-carboxyglutamyl residues. Subcellular localization studies have shown that the carboxylase activity is associated with rough endoplasmic reticulum and appears to be an integral membrane protein (Carlisle and Suttie, 1980). Most attempts to purify the carboxylase have not been successful although de Metz *et al.* (1981) have recently reported some progress. The molecular mechanism of the carboxylation and the role of vitamin K in the reaction are not understood. However, evidence suggests that the epoxidation of the vitamin is in some way linked to the carboxylation event (Carlisle and Suttie, 1980; Suttie *et al.*, 1980a; Larson *et al.*, 1981).

Standard assays for vitamin K-dependent carboxylase activity have generally used precursors of vitamin K-dependent clotting factors isolated from vitamin K-deficient rat liver microsomes as substrates. Soute *et al.* (1981) have prepared a peptide substrate for vitamin K-dependent carboxylase by limited proteolysis of bovine descarboxyprothrombin. This peptide, called Fragment-Su, is composed of amino acids 13–29 of descarboxyprothrombin and has an apparent K_m in the micromolar range, approximately 100-fold lower than intact descarboxyprothrombin. The difficulty in isolating precursors and the problems associated with manipulating the concentration of an endogenous substrate have spurred the search for a simple, synthetic peptide to act as substrate (Suttie *et al.*, 1979). A number of low-molecular-weight peptides have been synthesized for use as carboxylase substrates (Suttie *et al.*, 1979; Rich *et al.*, 1981). A pentapeptide, which consists of residues 5–9 of the uncarboxylated bovine prothrombin precursor (viz. Phe-Leu-Glu-Glu-Val), is the prototype peptide used as substrate for the carboxylase (Suttie *et al.*, 1979) and has an apparent K_m in the millimolar range (Soute *et al.*, 1981).

Synthetic peptides have also been used in comparative studies of carboxylase substrate specificity (Houser *et al.*, 1977; Esnouf *et al.*, 1978; Suttie *et al.*, 1980b). The vitamin K-dependent carboxylase seems to prefer but not require adjacent glutamyl residues (Rich *et al.*, 1981). It is not clear whether the primary sequence around the carboxylated residue is critical for efficient carboxylation (Houser *et al.*, 1977; Suttie *et al.*, 1979; Rich *et al.*, 1981) although a hydrophobic environment appears to be most effective. Rich *et al.* (1981) have reported that peptides which have a high potential for helicity are the best substrates, suggesting that tertiary structure may also be of importance.

C. Calcium-Binding Characteristics of Prothrombin

The calcium-binding and calcium-dependent phospholipid-binding characteristics of most of the Gla-containing plasma proteins appear to be quantitatively similar (Nelsestuen, 1976; Nelsestuen *et al.*, 1978). The number of calcium-binding sites of these proteins has been estimated to be 6 (Prendergast and Mann, 1977; Bajaj *et al.*, 1975; Nelsestuen *et al.*, 1975) to 10 (Henriksen and Jackson, 1975) for prothrombin and 20 for Factor X (Henriksen and Jackson, 1975; Lindquist and Hemker, 1978). The similarities in calcium- and phospholipid-binding characteristics are not unexpected in light of the extensive sequence homology observed in the amino termini of the Gla-containing plasma proteins (see Fig. 1). Most studies of the kinetics of calcium-binding and calcium-dependent phospholipid binding have used prothrombin or the isolated prothrombin fragment-1 (residues 1–156 of prothrombin) as an investigative model. For this reason, structural and calcium-binding characteristics of prothrombin will be considered here as a prototype of other Gla-containing plasma proteins.

The complete amino acid sequence of bovine prothrombin has been determined (Magnusson *et al.*, 1975) and two preliminary reports on the crystal structure have been presented (Aschaffenburg *et al.*, 1977; Olsson *et al.*, 1980). However, the complete three-dimensional structure of the molecule has not been determined. Prothrombin is composed of 582 amino acids including 10 Gla

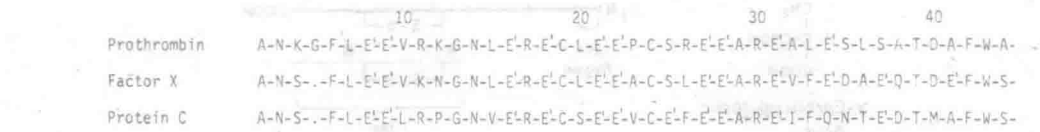

FIG. 1. Amino terminal sequences of bovine prothrombin (Magnusson *et al.*, 1975), Factor X light chain (Enfield *et al.*, 1975), and protein C (Fernlund and Stenflo, 1980a). Dots in the sequence denote the presence of a gap introduced for the purpose of alignment. E' denotes γ-carboxyglutamic acid. Modified from and numbered according to Davie (1980). The single letter code for the amino acids is as follows: A=Ala, D=Asp, E=Glu, F=Phe, G=Gly, H=His, I=Ile, K=Lys, L=Leu, M=Met, N=Asn, P=Pro, Q=Gln, R=Arg, S=Ser, T=Thr, V=Val, W=Trp, Y=Tyr.

residues and three complex carbohydrate chains. During activation of prothrombin to thrombin the NH_2-terminal peptide consisting of 156 residues is cleaved (Suttie and Jackson, 1977) (see Fig. 2). This peptide, known as fragment-1, contains all 10 of the Gla residues (Magnusson *et al.*, 1974) and two of the carbohydrate chains (Nelsestuen and Suttie, 1972; Magnusson *et al.*, 1975) of the intact prothrombin. Removal of the majority of sugar residues from prothrombin apparently does not alter its calcium-binding activity (Nelsestuen and Suttie, 1972).

Several lines of evidence indicate that prothrombin binds to phospholipid dispersions in the presence of calcium (Papahadjopoulos and Hanahan, 1964; Esnouf and Jobin, 1965; Barton and Hanahan, 1969; Nelsestuen, 1978) and that activation of prothrombin to thrombin is accelerated in the protein:calcium:phospholipid complex (Milstone, 1965). The phospholipid binding ability of prothrombin has been shown to be restricted to the fragment-1 region of prothrombin (Gitel *et al.*, 1973; Benson *et al.*, 1973) and acarboxyprothrombin does not bind phospholipid (Esmon *et al.*, 1975). The exact chemical nature of the phospholipid mixture does not appear to be critical (Bangham, 1961; Papahadjopoulos *et al.*, 1962) but optimal binding has been shown to occur between prothrombin–calcium complex and phospholipids with a specific negative surface charge density (Papahadjopoulos *et al.*, 1962; Dombrose *et al.*, 1979).

The 10 Gla residues in prothrombin fragment-1 appear to be required for maximum efficiency of calcium and phospholipid binding (Esmon *et al.*, 1975). When glutamyl residues are substituted for Gla residues both calcium and phospholipid binding are decreased (Esnouf and Prowse, 1977; Friedman *et al.*, 1977). Tertiary structure of the protein may contribute to the spatial proximity of Gla residues and thus influence the strength of calcium binding at a particular binding site. However, data obtained (Marsh *et al.*, 1980, 1982) with small

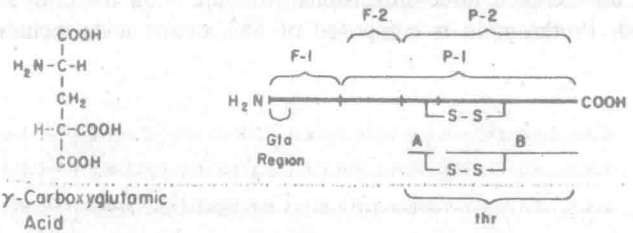

FIG 2. Structure of γ-carboxyglutamic acid (Gla) and a diagrammatic representation of the prothrombin molecule. Proteolysis of prothrombin by thrombin and factor X will cleave prothrombin into the specific large peptides shown: fragment-1 (F-1), fragment-2 (F-2), prethrombin-1 (P-1), prethrombin-2 (P-2), and thrombin (thr). For details of the activation of prothrombin and thrombin see Suttie and Jackson (1977). Modified from Suttie (1980a).

synthetic peptides suggest that the dianion contributed by Gla is sufficient to chelate calcium with an affinity close to that of the native protein.

Calcium binding to prothrombin fragment-1 has been described as consistent with both positive cooperative (Nelsestuen, 1976; Prendergast and Mann, 1977) and multiclass kinetics (Benson and Hanahan, 1975; Jackson, 1980). It has been suggested (Brenkle *et al.*, 1980; Jackson, 1980) that these data are not in conflict but that the complex calcium-binding behavior of isolated prothrombin fragment-1 depends upon the protein concentration at which the binding studies are done. Prothrombin fragment-1 undergoes a calcium-dependent self-association (Jackson *et al.*, 1979; Brenkle *et al.*, 1980; Jackson, 1980) and this protein–protein interaction may be responsible for the cooperative calcium-binding kinetics observed. However, self-association may not occur in the intact prothrombin molecule (Nelsestuen *et al.*, 1980b) so the protein–protein interactions may not be physiologically relevant.

Several lines of evidence suggest that a conformational change occurs in both isolated prothrombin fragment-1 (Nelsestuen, 1976; Prendergast and Mann, 1977; Blanchard *et al.*, 1980; Carlisle *et al.*, 1980; Nelsestuen *et al.*, 1980a,b) and in intact prothrombin (Bloom and Mann, 1978; Benarous *et al.*, 1980; Blanchard *et al.*, 1980; Nelsestuen *et al.*, 1980b) upon binding of calcium and that this change may involve an increase in helicity (Bloom and Mann, 1978; Nelsestuen *et al.*, 1980a). The conformational change occurs in the presence of millimolar concentrations of either calcium, Mg^{2+}, or Mn^{2+} and has been measured by a number of techniques including fluorescence quenching (Prendergast and Mann, 1977) and circular dichroism (Nelsestuen, 1976; Bloom and Mann, 1978). However, fluorescence quenching occurs prior to fragment-1:phospholipid binding and metals other than calcium, e.g., Mg^{2+}, can cause fluorescence quenching without bestowing phospholipid binding properties on the fragment-1:metal ion complex (Nelsestuen, 1976).

In summary, it appears that calcium binding by prothrombin is mainly due to a chelation of the cation by the two anionic carboxyl oxygens contributed by the Gla residues. Available evidence suggests that a conformational change occurs following binding of metal ions to prothrombin fragment-1 (Prendergast *et al.*, 1980). Whereas other metal ions may bind and induce a conformational change in protein structure, phospholipid binding will occur only in the presence of calcium. It has been proposed (Nelsesteun, 1976, 1978; Marsh *et al.*, 1980) that this calcium-dependent conformational change is the rate-limiting step in phospholipid binding to prothrombin.

D. Plasma Proteins Containing γ-Carboxyglutamic Acid

Seven plasma proteins containing γ-carboxyglutamic acid have been described: prothrombin (Downing *et al.*, 1975; Magnusson *et al.*, 1975), factor X