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THE PURIFICATION AND PROPERTIES OF AN INTESTINAL CALCIUM BINDING PROTEIN FROM THE BABOON

JEFFREY MAX BLOCH

A dissertation submitted to the Faculty of Science of the University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 1985.

ABSTRACT

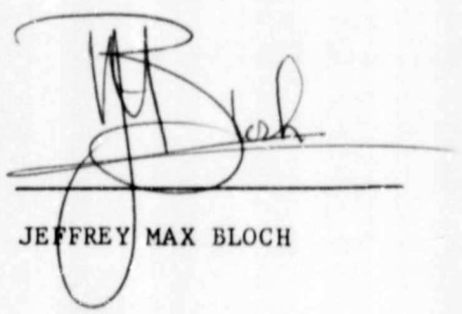
Intestinal calcium binding proteins have been studied in many species of mammals and birds. There are uncertainties as to the vitamin D-dependency and molecular weight of such proteins, especially in higher mammals. This dissertation describes the existence of a vitamin D-dependent calcium binding protein (CABP) in baboon intestine. The protein was partially purified and characterised by a combination of ammonium sulphate fractionation, heat and pH precipitation, gel permeation chromatography, ion-exchange chromatography, non-denaturing and sodium dodecyl sulphate (SDS) gel electrophoresis, counter-ion electrophoresis and high pressure liquid chromatography (HPLC). The advantages and disadvantages of these procedures are also discussed.

The vitamin D-dependency of the baboon intestinal CaBP was shown by dietary experiments. The presence of calcium binding activity was shown by gel permeation chromatography and counter-ion electrophoresis in these experiments.

The molecular weight of the protein was determined and shown to be approximately 11 200-11 900 daltons by gel permeation chromatography. Two low molecular weight components of approximately 11-12 500 daltons were evident on SDS polyacrylamide gel electrophoresis. The CABP activity of these proteins was however not established.

DECLARATION

I, Jeffrey Max Bloch, declare that this dissertation is my own, unaided work. The work reported in this dissertation was carried out at the University of the Witwatersrand in the research laboratories of the Department of Medical Biochemistry, Medical School and the Metabolic Research Unit, Baragwanath Hospital, Johannesburg, South Africa.



JEFFREY MAX BLOCH

30th day of the ninth month, 1985.

DEDICATION

In loving memory of my father,

HEINZ JACOB BLOCH

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1. Dr. F.P. Ross (Paddy) for his invaluable assistance, encouragement, persistent motivation and friendship which he so generously offered during this project.

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LIST OF ABBREVIATIONS

A280/A540	:	Absorbance at 280mm or 540mm
BSA	:	Bovine serum albumin
CaBP	:	Calcium binding protein
CaBP Assay	:	Calcium binding protein assay
Ca*Pr/Ca*R	:	Ratio of labelled calcium bound to protein to that bound to Chelex resin
$CaCl_2$	:	Calcium chloride
$^{45}CaCl_2$	:	Radioactive calcium chloride
cpm	:	Counts per minute
EDTA	:	Ethylenediaminetetra-acetic acid
HPLC	:	High pressure liquid chromatography
$(NH_4)_2SO_4$	:	Ammonium sulphate
NaCl	:	Sodium chloride
SDS	:	Sodium dodecyl sulphate
Tris	:	Tris (hydroxymethylaminomethane)
Temed	:	$N_1N_1N_1N_1$ - Tetra methylethylenediamine
$1,25(OH)_2D_3$	:	1,25-dihydroxyvitamin D_3
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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Calcium plays a central role in a wide range of both intracellular and extracellular events (Rasmussen et al, 1976; Means and Dedman, 1980, Berridge, 1984; Rasmussen and Barrett 1984). It has been hypothesised that regulation of cell function is controlled by two messengers, namely, calcium ions and cyclic AMP (Rasmussen and Barret, 1984). It has become evident that in such regulation calcium ions and cyclic AMP often function together. Although the molecular and cellular mechanisms involved in the initiation, propagation, reception and termination of the cAMP message have been known for some time, it has recently become evident that the calcium messenger system is more complex than the cyclic AMP system (Sutherland et al, 1968; Rasmussen and Clayberger, 1979).

Intracellular regulation can be accompanied by both dynamic organelle release and sequestration of calcium and by specific calcium active transport mechanisms located in the plasma membrane (Moore and Dedman, 1982a; Carafoli 1984). All eukaryotic and probably all prokaryotic cells maintain the concentration of free calcium ions in the cytosol at about 10^{-7}M while at rest (Kretsinger, 1977). In contrast the extracellular calcium concentration is at approximately 10^{-3}M. Following a stimulus, the concentration of calcium ions in the cell rises briefly to about 10^{-5}M and then returns to resting concentrations. The plasma membrane of the cell not only plays a role in maintaining cellular calcium homeostasis, but has a vital function in stimulus-response coupling when the cell employs the calcium messenger system. Calcium binds to the inner surface of the membrane in small amounts and considerably more is bound to the glycocalyx (Tan and Tashjian, 1981; Fleckenstein, 1983).

There also exists a relatively large pool of nonionic but exchangeable calcium within the mitochondrial matrix space in exchange with a small pool of free calcium ions within the same space (Cheung et al, 1981). When activation of the cell by the specific extracellular messenger occurs, specific calcium channels may open up, either in a transient or a sustained manner, to allow the ionised calcium to enter down a steep concentration gradient (Baker, 1976). In addition to these events occurring at the plasma membrane, two other membranes determine the concentration of calcium, namely, the membrane of the endoplasmic reticulum and the inner mitochondrial membrane. Both of these have pump-leak systems specifically arranged to affect the active extrusion of calcium from and the passive leak of calcium back into the cytosol of the cell. Each of these two membranes has a unique role to play in the cell. The mitochondrial membrane pump-leak system serves two important functions. Firstly, at the low ionic concentration of calcium, it is responsible for stabilising the calcium ion concentration. Secondly, at high concentrations of calcium ions, it serves as a reservoir for storing calcium during periods of excessive cellular calcium accumulation (Rasmussen and Waisman, 1982). The endoplasmic reticulum serves as a source of calcium for the initial phase of cellular activation in many cells (Ebashi et al, 1978; Bolton, 1979; Ronning et al, 1982; Nishizuka, 1984a,b).

Calcium has been postulated as being a second messenger which transmits its information by binding to calcium-modulated proteins and thereby altering their conformation and function (Rasmussen, 1970). Calcium modulated proteins can be defined by two characteristics:

I) They occur in the cytosol or on membranes facing the cytosol;

II) They bind calcium when there is a transient increase in calcium ion

concentration in the cell and release calcium as the calcium ion concentration returns to basal levels (Rasmussen, 1970). Examples of calcium modulated proteins are calmodulin (Means and Dedman, 1980; Dedman et al, 1979; Cheung, 1980; Klee et al, 1980), troponin C (Levine et al, 1977), parvalbumin (Pechere et al, 1973; Lehky et al, 1974; Moeschler et al, 1979), myosin light chain (Weeds and McLachlan, 1974; Tufty and Kretsinger, 1975) and brain S-100 (Hyden, 1974; Callissano et al, 1975). A particular characteristic of these proteins is that they are said to be homologous (Goodman et al, 1979). They have evolved from a common ancestral binding domain, known as the 'EF-hand', which was first described in the crystal structure of parvalbumin (Meows and Kretsinger, 1975). Another group of proteins which share this homology, but which are not functionally modulated by calcium, are the vitamin D-dependent calcium binding proteins (CaBPs). In particular the existence of the 'EF hand' has been shown recently in the crystal structure of bovine intestinal CaBP (Szebenyi et al, 1981).

In a recent review on calcium messenger system, two classes of receptor proteins were described (Rasmussen and Barret, 1984). Firstly, there exist true calcium receptor proteins, one of which belongs to the group of homologous proteins. For example calmodulin has no intrinsic enzymatic activity, but when it binds calcium it undergoes a conformational change that alters its association with other proteins and response elements. This ultimately causes a change in the activity of these response elements. Secondly, calcium regulated enzymes such as phospholipid-dependent calcium-activated protein kinase exist which bind calcium directly (Adelstein et al, 1980; Cheung et al, 1981; Castanga et al, 1982; Cox et al, 1984).

The binding of ionic calcium which appears to be specific and physiologically significant, has been reported for a number of different CaBPs. The first mediator of calcium signals described was the troponin complex of striated muscle. It was shown that the troponin complex was composed of three subunits of which troponin C was the one which interacted directly with calcium. Troponin C is reported to have a 50% homology in amino acid composition to calmodulin (Wang and Waisman, 1980). Troponin C has a molecular weight of 17 846 daltons and contains four calcium binding sites. Troponin C has two sites which are capable of binding magnesium (pkd = 3,5), two sites capable of binding calcium (pkd = 6,7) . Both two sites capable of binding either calcium or (pkd = 8,7) magnesium (pkd = 3,5). (Potter and Gergely, 1975). The affinity of Troponin C for calcium is greatly enhanced by its interaction with Troponin I. There is no co-operativity in calcium binding. During a relaxation - contraction-relaxation cycle the calcium concentration ranges from approximately 10^{-8}M to approximately 10^{-5}M. The magnesium concentration in the sarcoplasm is in the range 10^{-6}M to 10^{-3}M, hence the high affinity (calcium or magnesium) site would appear to be occupied by a divident cation at all times. The calcium binding domains exhibit 70% sequence homology to calmodulin calcium binding domains. As in the case of calmodulin, the α-helical content of troponin C increases when calcium binds (Levine et al, 1977). This leads to troponin C interacting with a specific intracellular protein troponin I. Reversal of contraction occurs upon calcium sequestration by the sarcoplasmic reticulum. Calcium-dependent regulation of skeletal muscle has been adequately described (Kretsinger and Barry, 1975).

A second example of an important protein that binds calcium is calmodulin. This protein is a monomer of molecular weight 17 000 daltons. Its tertiary structure is such that it contains four nearly

equivalent calcium binding domains, two of which are high affinity binding sites showing dissociation constants of approximately $1 - 4 \times 10^{-6}$M. The other two sites show slightly lower affinity and can accept magnesium under certain conditions (Klee et al, 1980). Binding of calcium causes the molecule to increase its α-helical content and expose a hydrophobic binding region. This region appears to be utilised by various calmodulin inhibitors including some neuroleptic drugs and analgesics (Wong and Waisman, 1980; Cheung, 1980; Klee et al, 1980). Calmodulin is ubiquitous in both plant and animal cells and it thus seems likely that multiple roles exist for this protein.

A third member of the family of homologous proteins is parvalbumin. Parvalbumins are acidic muscle sarcoplasmic proteins having a molecular weight of 12 000 daltons. These proteins are found in abundance in fast skeletal muscle of lower vertebrates (Pechere et al, 1973). They are not known to have any established function in muscle, although some experiments suggest they may be able to inhibit ATPase activity by lowering the calcium concentrations through chelation (Moeschler et al, 1979).

Another example of an homologous CaBP which is unique to the nervous system and whose function remains unknown, is S-100. It is found in the glial cells of both vertebrates and invertebrates and only a portion of it is membrane bound. However, immunofluorescent labelling studies have shown that some S-100 is associated with and supposedly involved in the formation of the post-synaptic depression of neurons. When calcium binds to S-100 it induces a conformational change that exposes hydrophobic groups and thus makes the protein capable of interacting with liposomes or membranes and of changing their permeability to some cations

(Callissano et al, 1975). A final member of the group of homologous proteins is the vitamin D-dependent intestinal CaBP which will be discussed in detail later.

There are also CaBPs which do not belong to the homologous group of proteins. These proteins exhibit calcium binding properties of both high and low affinity. Examples of the former are calsequestrin (Han and Benson, 1970; Otswald and MacLennan, 1974) and calcium-activated ATPase of vertebrate sarcoplasmic reticulum (MacLennan and Holland, 1975). Calcium activated neutral proteases (Orrego, 1964; Guroff, 1964; Bush et al, 1972; Dayton et al, 1975; Small and Sobieszek, 1977; Libby and Goldberg, 1978; Nelson and Traub, 1981), transglutaminases (Griffin et al, 1978; Cohen et al, 1981) and calcemedins (Moore and Dedman, 1982b) are examples of the latter.

There are also a number of extracellular CaBPs which, with the exception of α-amylase, bind calcium rather weakly (Kretsinger, 1976). Some of the most important extracellular CaBPs are involved in the blood clotting system. Four plasma glycoproteins, clotting factors II (prothrombin), VII, IX and X are known to require vitamin K for their activation (Davie and Fujikawa, 1975; Stenflo, 1976). These proteins contain γ-carboxyglutamic acid residues which enable them to bind calcium in solution (Nelsteun and Suttie, 1972; Stenflo and Ganrot, 1973; Benson et al, 1973; Bajaj et al, 1974; Henriksen and Jackson, 1975). The calcium binding properties of prothrombin and the other vitamin K-dependent proteins are the result of vitamin K-action and are crucial for phospholipid binding and subsequent biological activity.

Blood coagulation involves a series of consecutive conversions of plasma zymogens to the corresponding active serine endopeptidases by limited proteolysis. The last reaction in the series is the activation of prothrombin to thrombin which in turn converts soluble fibrinogen to insoluble fibrin. The Y-carboxyglutamic acid residues are all situated near the N-terminal ends of these glycoproteins. These residues were identified in the peptide containing residues 4-10 in prothrombin (Stenflo et al, 1974). After degradation of this peptide with aminopeptidose M and carboxypeptidase B, a tetrapeptide was isolated. Protein, nuclear magnetic resonance spectroscopy and mass spectrometry showed that each of the two glutamic acid residues had one extra carbonyl group on their Y-carbon atoms. The tetrapeptide thus contained two residues of Y-carboxyglutamic acid (Gla), a previously unkown amino acid (Nelsteun et al, 1974). These so-called 'Gla' residues are known to confer upon these proteins calcium binding properties. A fifth plasma glycoprotein which also contains Y-carboxyglutamic acid residue has been found, but as yet no function for it has been shown (Stenflo, 1976).

In contrast to the group of extracellular CaBPs, a number of CaBPs exist which show specific tissue distribution and are induced by vitamin D. Mammals, birds and other vertebrates synthesise such proteins on induction by an active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).

Vitamin D is a secosterol required by all mammals for the optimal intestinal absorption of calcium. It is known that vitamin D does not exert its effects on calcium metabolism directly, but must first undergo an obligatory two step metabolism to $1,25(OH)_2D_3$, the biological active form of the steroid (Norman, 1971; Avioli and Haddad, 1973; Kodicek, 1974; DeLuca, 1974; DeLuca and Schnoes, 1983).

The first step is an hepatic 25-hydroxylation reaction and a second renal 1α-hydroxylation. Figure 1 shows a schematic representation of vitamin D metabolism. The most important dihydroxylated metabolite of vitamin D, namely, $1,25(OH)_2D_3$ has been clearly linked with the kidney, as this organ functions as the endocrine gland for secretion.

A number of the biochemical properties of the 25-hydroxylase enzymes involved in the hepatic reaction have been described. It is now generally accepted that the liver is the tissue responsible for the production of 25(OH)D (Horsting and DeLuca, 1969). The 25-hydroxylation process was found to be stimulated by molecular oxygen in the presence of NADPH (Horsting, 1970). The same author showed that the enzyme was present in the liver mitochondrial fraction. It was subsequently reported, that the 25-hydroxylase activity was associated with the liver microsomal and not the mitochondrial fraction (Bhattacharyya and DeLuca, 1974). Evidence suggests that vitamin D metabolism by the liver occurs via a mixed function oxidase system, which is also a common means of hydroxylating many steroids and drugs (Cinti et al, 1976). Figure 2 shows the sequential organ-specific hydroxylations of vitamin D that are required for the conversion to the biologically active hydroxylated metabolite $1,25(OH)_2D_3$.

A most significant development in the understanding of the mechanism of vitamin D action concerns the fact that the renal production of $1,25(OH)_2D_3$ may be regulated in response to changing endocrinological or physiological signals. An important aspect of the possible regulation of renal 25(OH)D-1-hydroxylase is that the organism can regulate the amount of $1,25(OH)_2D_3$ in accordance with its calcium needs (Norman, 1979).

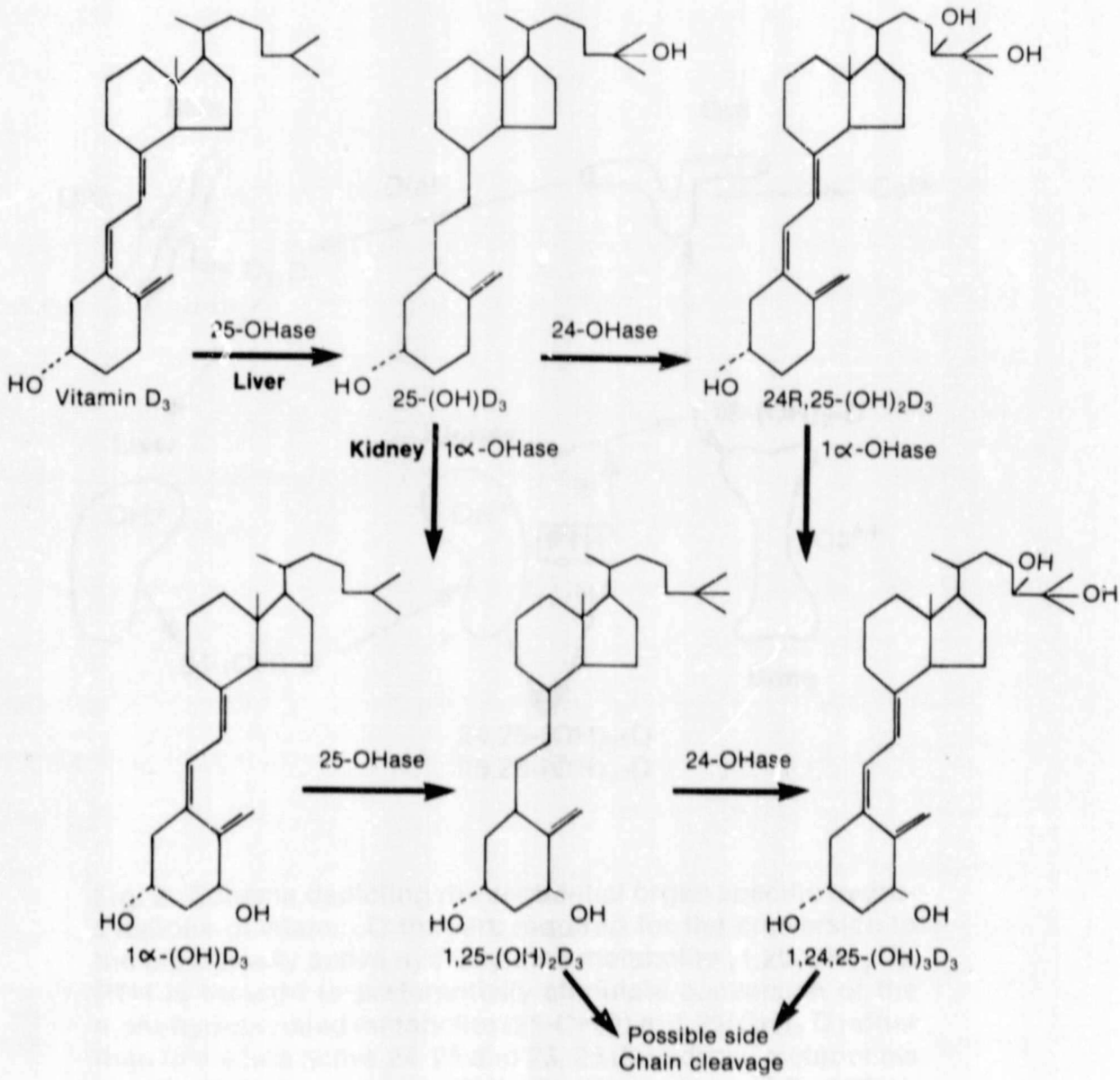


Fig. 1: Metabolism of vitamin-D_3 showing enzymatic steps and hydroxylation steps (Haussler, et al. (1977)).

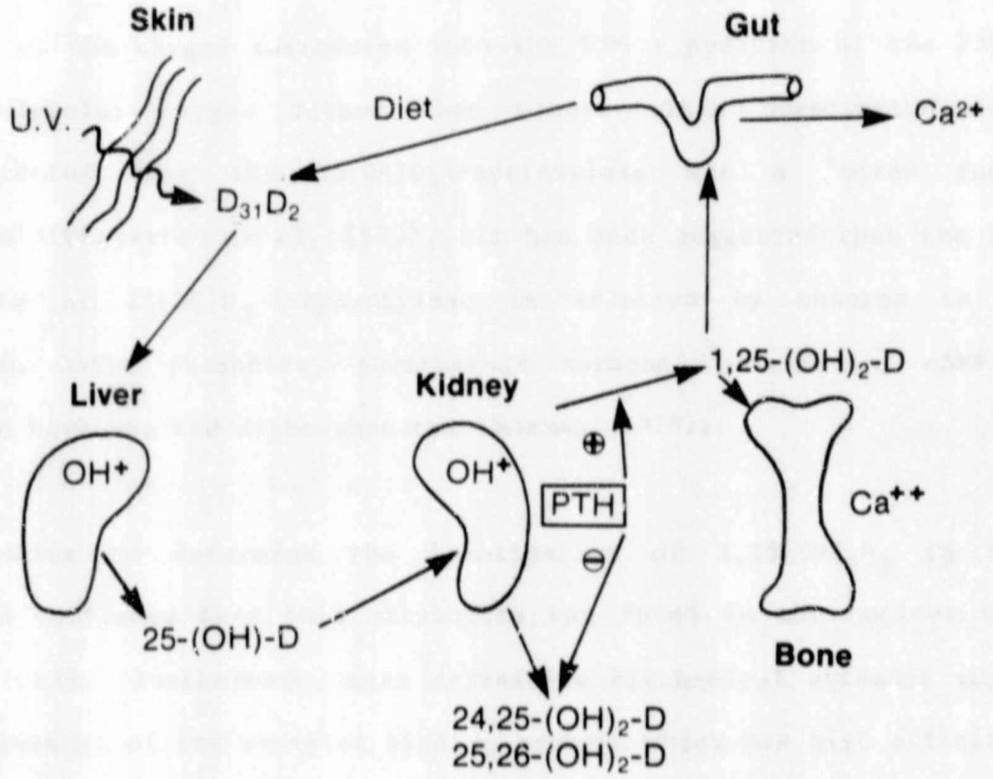


Fig. 2: Schema depicting the sequential organ specific hydroxylations of vitamin D that are required for the conversion to the biologically active hydroxylated metabolite ($1,25(OH)_2$ D). PTH is thought to preferentially stimulate conversion of the monohydroxylated metabolite (25-OHD) to $1,25(OH)_2$ D rather than to the less active 24, 25 and 25, 26 di-hydroxy metabolites ($24,25\text{-}(OH)_2$ D and $25,26\text{-}(OH)_2$ D). (Rickenberg, H.B. (1974)).

It was initially suggested, that 1-hydroxylase enzyme activity was associated with either the crude nuclear or heavy mitochondrial fraction of the cell (Fraser and Kodicek, 1970). It has now been clearly shown that the 1-hydroxylase enzyme is exclusively associated with the mitochondrial fraction of the kidney (Gray et al, 1972; Midgett et al, 1973). Through the use of ^{18}O, it has been demonstrated that the source of the oxygen introduced into the 1α - position of the $25(OH)D_3$ was molecular oxygen rather than water. This observation clearly demonstrated that the $25(OH)D_3$1-hydroxylase was a 'mixed function oxidase' (Ghazarian et al, 1973). It has been suggested that the enzyme activity of $25(OH)D_3$-1-hydroxylase is affected by changes in serum calcium, serum phosphate, parathyroid hormone, calcitonin, cAMP, pH, steroid hormones and diphosphonates (Norman, 1979).

Studies to determine the localisation of $1,25(OH)_2D_3$ in target tissues confirmed that this metabolite was found in the nucleus of the target cell. Furthermore, more definitive biochemical evidence supports the existence of the receptor binding protein which has high affinity for $1,25(OH)_2D_3$ (Stumpf et al, 1980, 1981, 1982). The tissue distribution of receptor proteins for $1,25(OH)_2D_3$ has been thoroughly documented and these proteins have been shown to exist in a variety of mammalian and avian tissues including the intestine, colon, parathyroid gland, bone, pancreas, pituitary, eggshell gland (hen), choriollantoic membrane, ovary, yolk sac (rat), parotid gland, skin, placenta, testes, uterus, mammary gland, thymus of higher animals and in several non-mammalian/avian tissues including, the skin, intestine, kidney, liver, gill, brain, spinal chord, pituitary and corpuscles of the stannius of the fish (Norman et al, 1982).

The most thoroughly characterised and studied receptor for $1,25(OH)_2D_3$ is that from the chick intestinal mucosa. Early reports suggested that the receptor had a molecular weight of between 47 000 by Brumbaugh and Haussler, (1975) and 70 000 daltons (Wecksler, 1978). More recent studies have shown the molecular weight of the occupied $1,25(OH)_2D_3$ receptor to be of the order of 90 - 100 000 daltons (Bishop et al, 1982). The receptor has also been shown to have a stokes radius of 36 - 38 Å. It has now been demonstrated that the molecular weight of the occupied intestinal receptor for $1,25(OH)_2D_3$ is approximately 68 000. (Pike and Haussler, 1980; Pike et al, 1983).

The specifity of binding of $1,25(OH)_2D_3$ has been thoroughly examined (Eisman and DeLuca, 1973; Procsal et al, 1975, Kream et al, 1977; Wecksler and Norman, 1978, 1979, 1980). Structural specificity of ligand binding has shown that the 1-hydroxyl and 25-hydroxyl groups are the most critical for efficient receptor recognition, whereas, the 3 β-hydroxyl is only 10% as important (Procsal et al, 1975; Wecksler et al, 1978).

It has not been uniformly agreed that there exists a temperature-dependent 'activation' or transformation of the $1,25(OH)_2D_3$ receptor complex. Originally Brumbaugh and Haussler, (1974a) and more recently Feldman et al, (1979) stated that there was a mandatory temperature-dependent activation step required for the association of the $1,25(OH)_2D_3$ - receptor complex with chromatin. This process was, however, not accompanied by a change in the sedimentation coefficient of the receptor (Brumbaugh and Haussler, 1974a,b, 1975; Wecksler and Norman, (1978), Wecksler and Norman, 1980).

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