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PROSPECTS

Vitamin D Hydroxylases

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Abstract There are three mixed function oxidases which catalyze hydroxylations of vitamin D and its derivatives. These include the hepatic mitochondrial or microsomal vitamin D_3-25-hydroxylase and the two renal mitochondrial enzymes which further hydroxylate 25-hydroxyvitamin-D_3 (25-OH-D_3) to form 24R,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the primary steroid hormonal derivative of vitamin D_3. All three enzymes are cytochrome P450 dependent. The two renal mitochondrial enzymes are regulated, usually in a reciprocal fashion. The intracellular signalling systems involved in this regulation include 1,25$(OH)_2D_3$ itself and both protein kinases A and C. Recent progress has been made in the purification and cloning of the vitamin D_3-25-hydroxylase and the 25-OH-D_3-24-hydroxylase. When the 25-OH-D_3-1-hydroxylase is purified and cloned, efforts which have thus far been frustrated by its low abundance, fertile new ground for the study of the regulation of vitamin D metabolism at the molecular level will be opened up.

Key words: 1,25-dihydroxyvitamin D_3, 24R,25-dihydroxyvitamin D_3, 25-hydroxyvitamin-D_3, cytochrome P450, kidney mitochondria

In the field of study of the steroid hormones in general and vitamin D in particular, much attention has been directed to the objectives of identifying target sites; determining the biological effect of the hormone of interest at that target site; and elucidating the molecular mechanism of action of the hormone in bringing about the biological effect. Somewhat less activity has been directed towards the other major point at which the action of steroid hormones is controlled, i.e., their rate of synthesis. This paper is a status report on the understanding we currently have of the enzymes whose activities determine the rate of synthesis, and therefore, ultimately, the biological activity, of the vitamin D related steroids, principally 1,25-dihydroxyvitamin D_3.

The known active steroid hormonal form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is produced in the kidney from 25-hydroxyvitamin D_3 (25-OH-D_3), which in turn is produced in the liver from the parent vitamin D compound and represents the major circulating form of the hormone [1,2]. Vitamin D itself is the product of a reaction which occurs in the skin in response to ultraviolet light. The kidney is also capable of producing 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$], as are several other tissues as detailed below. 1,25$(OH)_2D_3$ can also be produced by certain cell types in culture such as activated macrophages [3] and bone cells in culture [4]. These central pathways of vitamin D metabolism are depicted in Figure 1.

In contrast to many other steroid hormones, such as glucocorticoids, estrogens and androgens, whose rate of synthesis is regulated largely at the point of the cleavage of the side chain of cholesterol, the production of 1,25$(OH)_2D_3$ is regulated exclusively, as far as we know, at the last step in the pathway of its production, the 25-OH-D_3-1α-hydroxylase. Thus, the 25-OH-D_3-1α-hydroxylase has been the subject of investigation by several workers in the vitamin D field since its discovery over two decades ago. The related enzymes, 25-OH-D_3-24R-hydroxylase and vitamin D_3 25-hydroxylase have also been studied and will be reviewed here.

HISTORICAL PERSPECTIVE

The kidney was identified as the site of 1,25$(OH)_2D_3$ synthesis—and as a site of 24,25$(OH)_2D_3$ synthesis—over two decades ago [5,6]. The substrate for these reactions, 25-OH-D_3 had recently [7] been shown to be a product of the liver. In 1974, Ghazarian et al. [8] showed that a solubilized kidney mitochondrial preparation contained both cytochrome P450

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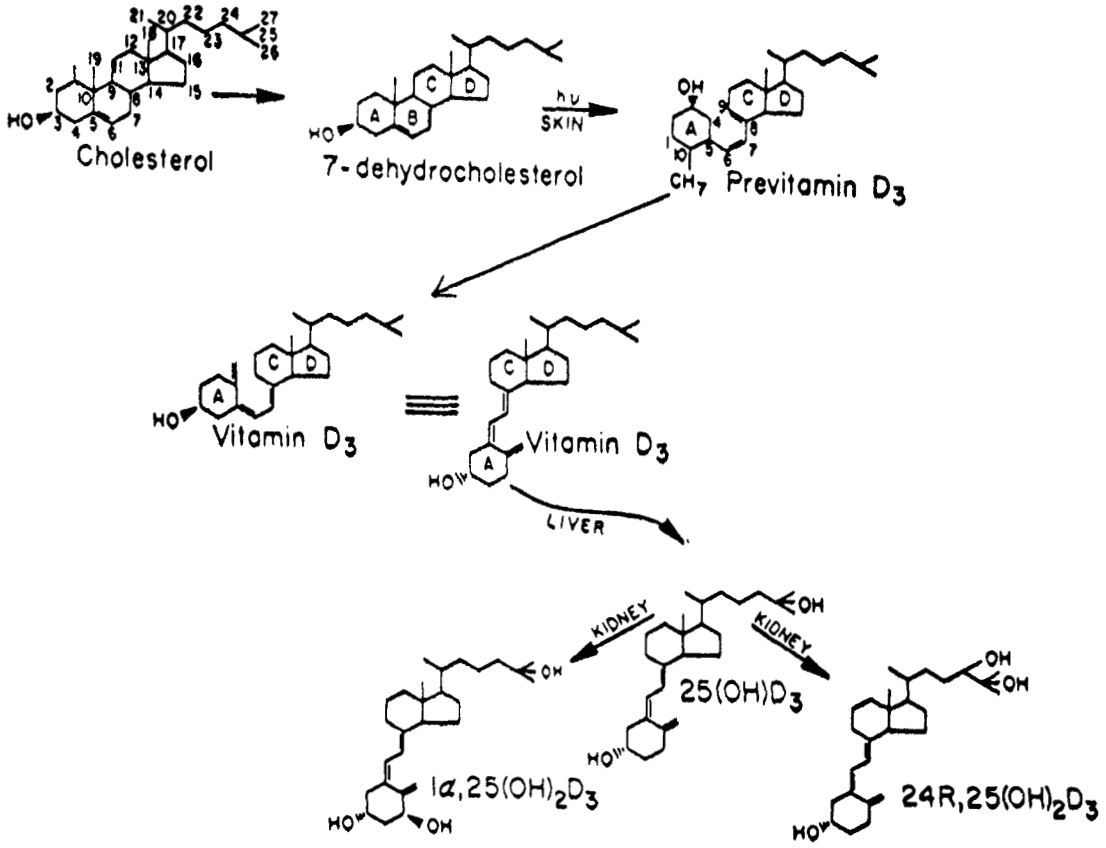

Fig. 1. The central pathway of vitamin D metabolism.

and 1-hydroxylase activity; in the same year Henry and Norman documented the relationship between the kidney mitochondrial cytochrome P450 and 1-hydroxylase activity by photochemical activation experiments [9]. The ensuing two decades have seen several occasional reports on the solubilization and reconstitution of 1-hydroxylase activity, which have in general tended to give credence to the early assumption that the 1-hydroxylase (and probably the 24-hydroxylase as well) are similar to classical mitochondrial mixed function oxidases which hydroxylate endogenous steroids. These enzymes are exemplified by the cholesterol side chain cleavage enzyme, one of the earliest recognized and most thoroughly studied of these enzymes whose prototypical organization is depicted in Figure 2. Although it has occasionally been speculated [e.g., 10] that 1- and 24-hydroxylase activity might exist in the same cytochrome P450 with its hydroxylation activity switched between the two positions by allosteric changes in the protein, recent physical separation of the two activities (see below) argues against this possibility.

Due to the very low amounts of enzyme present in kidney mitochondria, the purification of these putative components of the 1-hydroxylase has been a largely intractable problem and current status of efforts in this regard is described later in this paper. In the meantime, attention has focused on the regulation of 25-OH-$D_3$1- and 24-hydroxylase activity in whole animal studies and in cell culture.

REGULATION OF 25-OH-D_3 HYDROXYLATION

Numerous studies during the 1970's [see 1 for review] firmly established that one of the most powerful ways to influence the pattern of 25-OH-D_3 metabolism by the kidney or mitochondrial fractions therefrom is to alter the vitamin D status of the animal. Since this most easily accomplished in birds, the original discovery of the 1-hydroxylase [5,6], as well as the effect of vitamin D status on its activity, was made in young vitamin D deficient chicks. Although there

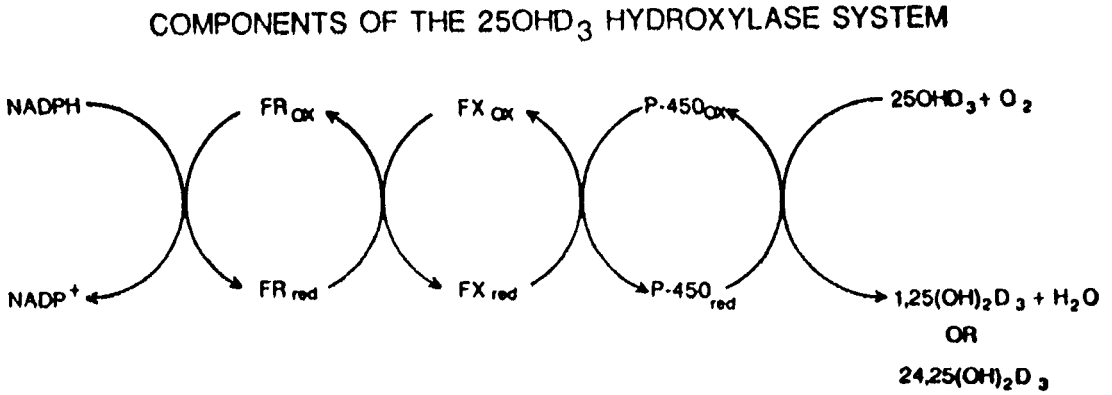

Fig. 2. General scheme of mitochondrial mixed function oxidases. The electrons for the reduction of molecular oxygen to H_2O and —OH (hence the term mixed function oxidase) are ultimately derived from NADPH, having been passed to cytochrome P450, an integral protein of the inner mitochondrial membrane, by ferredoxin reductase and ferredoxin. The latter is a 12–14 kDa iron sulfur protein of the mitochondrial matrix which can be envisioned to shuttle back and forth between the reductase and the cytochrome P450, which confers the substrate specificity and the specificity of the site and stereoconfiguration of the hydroxylation reaction.

have been reported quantitative variations from species to species, the following fundamental observations have held up in a variety of mammalian species: In the absence of vitamin D, the 1-hydroxylase is elevated to its maximal levels and very little, if any, $24,25(OH)_2D_3$ is produced by kidney tissue; in vitamin D repletion, 1-hydroxylase activity is repressed and 24-hydroxylase is induced, the latter effect being enhanced by high dietary calcium in whole animal studies. As shown in Figure 3 these effects can be attributed to the active form of the hormone, $1,25(OH)_2D_3$ when the direct effect on cultured kidney cells was tested [11].

The pattern of hydroxylation of 25-OH-D_3 is also influenced by cyclic AMP and calcium/phospholipid mediated pathways of intracellular signalling. In cell culture studies, activation of the former pathway leads to stimulation of $1,25(OH)_2D_3$ production and a dimmunition in the synthesis of $24,25(OH)_2D_3$ [12]. Activation of the calcium/phospholipid pathway has precisely the opposite effect [13] diminishing $1,25(OH)_2D_3$ synthesis and increasing that of $24R,25(OH)_2D_3$, although whether this is due to activated protein kinase C or subsequent changes in its subcellular distribution has not yet been established.

One way to determine the mechanism of these three major known regulatory pathways—$1,25(OH)_2D_3$, protein kinase A, and protein kinase C—is to determine the role of on-going protein synthesis in each. Table I summarizes these effects. The induction of the 24-hydroxylase by $1,25(OH)_2D_3$ is blocked by cycloheximide

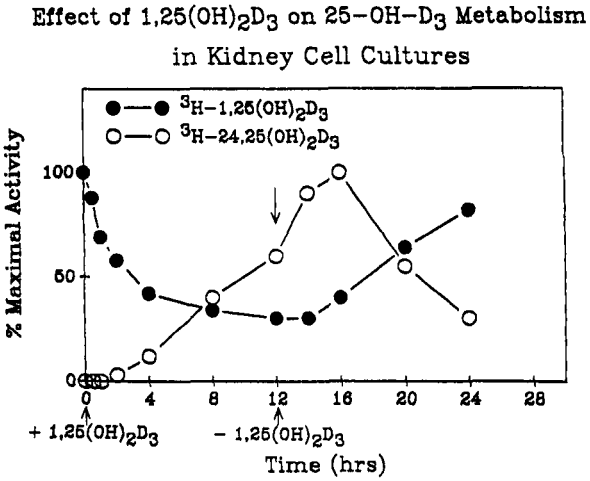

Fig. 3. Regulation of the hydroxylation of 25-OH-D_3 by $1,25(OH)_2D_3$. The amount of $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ produced from 25-OH-D_3 is shown as a function of time after the addition (at time 0) and removal (at 12 h) of $1,25(OH)_2D_3$ to primary cultures of chick kidney cells in serum free medium.

and, although the experiments are rather more difficult to carry out and interpret, the repression of the 1-hydroxylase by $1,25(OH)_2D_3$ probably also requires on-going protein synthesis. The decrease in 1-hydroxylation resulting from the activation of protein kinase C is independent of on-going protein synthesis, whereas its elevation in response to protein kinase A does require protein synthesis. Since the effects of the two protein kinases on 24-hydroxylase activity can only be assessed in the presence of $1,25(OH)_2D_3$ to induce the activity and since cycloheximide blocks this induction, the role of protein synthe-

TABLE I. Regulatory Mechanisms in 25-Hydroxyvitamin D Metabolism

	Synthesis of $1,25(OH)_2D_3$	Inhibited by cycloheximide	Synthesis of $24,25(OH)_2D_3$	Inhibited by cycloheximide
PKA	↑	+	↓	ND
PKC	↓	−	↑	ND
$1,25(OH)_2D_3$	↓	(+)	↑	+

sis in this process cannot be determined in this system.

Clearly the regulation of these two enzymes is multifactorial and complex; only the development of the appropriate molecular reagents will permit the elucidation of the interaction of the various intracellular signalling pathways governing the pattern of hydroxylation of 25-OH-D_3 in the kidney cell. The need to study the proteins involved in these hydroxylations and their genes has been recognized for some time. Attention has naturally focused on the cytochrome P450 components of these hydroxylases, as well as that of the vitamin D_3-25-hydroxylase, since this is where the specificity for hydroxylation lies. The current status with each of the three enzymes is described below.

It should be mentioned that some reports have implicated the ferredoxin component of the hydroxylation system in the regulation of 1-hydroxylation and 24-hydroxylation. While the data indicate that both renal [14] and adrenal [15] ferredoxin can be phosphorylated, at least in the case of the renal hydroxylation of 25-OH-D_3 the relationship between phosphorylation state and changes in hydroxylase activity has not yet been firmly established.

PURIFICATION OF THE HYDROXYLASES

Vitamin D_3-25-Hydroxylase

The liver is thought to be the principal site of the hydroxylation of vitamin D to 25-OH-D, although substantial activity can be shown to occur in other tissues as well. In rats in rabbits activity has been demonstrated in both microsomes and mitochondria [16–18] and both subcellular forms have been purified to homogeniety [17,19]. Cytochrome P450 with 25-hydroxylase activity has also been isolated from pig kidney microsomes [20]. In humans, only a mitochondrial form of the enzyme has been demonstrated and on this basis, as well as the fact that microsomes contain five fold less activity, some authors [21,22] have questioned the physiological significance of the microsomal enzyme. As recent successes in the cloning [23] and expression [24] of cDNAs coding for proteins with 25-hydroxylase activity (as well as other sterol side chain hydroxylation activity) is expanded, these issues should become more clearly resolved.

25-OH-D_3-24-Hydroxylase

Although there exist a number of reports of 24-hydroxylase activity solubilized from kidney mitochondria from chick and rat [25,26], little progress was made in its purification, due to its very low concentration, even when induced by vitamin D and high dietary calcium. Recently, however, the enzyme was purified to homogeneity from rat kidney mitochondria [27] and found to have a specific activity several thousand-fold higher than those previously reported. The purified enzyme displayed only 24-hydroxylase (and no 1-hydroxylase), strongly supporting the idea that the two hydroxylations are catalyzed by distinct cytochrome P450s. Even more recently, the same authors have cloned the cDNA for the rat kidney 24-hydroxylase cytochrome P450 [28], using antibodies raised against the purified enzyme. The cDNA, whose identity was confirmed by expression in COS cells, shows only limited homology with known mitochondrial cytochrome P450s, suggesting that it represents a novel class. This accomplishment can be considered a relatively major breakthrough in the field of vitamin D hydroxylases, although how much it will advance efforts to clone the 1-hydroxylase remains to be seen.

25-OH-D_3-1-Hydroxylase

The difficulties associated with the purification of the 24-hydroxylase, mainly very low abundance, apply even more so to the 1-hydroxylase. Again, there are numerous reports of its solubilization [e.g. 26,29,30] but little convincing evidence of its even partial purification. Furthermore, the activity of the solubilized preparations has been disappointly low (e.g., turnover number of 0.03 min^{-1} [31]). Early efforts [26] to purify the 1-hydroxylase were complicated by the finding by several laboratories [32] that us-

ing the HPLC methods of the time ($CHCl_3$ and hexane as the solvents) a non-enzymatically produced metabolite of 25-OH-D_3, 19-nor-10-oxo-25-OH-D_3, co-chromatographed with 1,25$(OH)_2D_3$. Modification of the solvent system used to separate the metabolites of 25-OH-D_3 overcame this difficulty but the problem of very low abundance has not yet been adequately resolved.

A report of antibodies specific for the enzyme appeared several years ago [33] but no further work characterizing or utilizing these antibodies has ensued. Recently, similar putative N-terminal sequences for both the 1-hydroxylase and the 24-hydroxylase from chick kidney mitochondria were published [34]; however, positive linkage of each sequence with its cognate enzymatic activity has not yet appeared.

FUTURE DIRECTIONS

There is no mystery about what is the most important objective in the area of vitamin D hydroxylases: the development of reliable reagents, i.e. antibodies and cDNAs, with which to study these enzymes, particularly the 25-OH-D_3-1-hydroxylase, on the molecular level. As indicated above some tangible progress has been made with respect to the mammalian vitamin D_3-25 hydroxylase and 25-OH-D_3-24-hydroxylases. Although there have been reports of similar progress with bovine and avian 1-hydroxylases, the evidence in this case is less convincing than for the 25- and 24-hydroxylases. It is likely that progress with the 1-hydroxylase will require very deft and possibly specialized purification techniques as well as application of PCR and/or RT-PCR techniques. For these latter approaches to be successful, appropriate cDNA libraries and mRNA populations must be chosen with care and confirmation of identity by expression of the specific hydroxylase activity will be required. In the case of antibodies, depending on how similar the two hydroxylases turn out to be, recognition specificity may be a complex issue to resolve. Although finally obtaining these reagents will have been difficult and at times frustrating, their availability will open up enormous new areas of enquiry not only into the fundamental mechanisms of the regulation of vitamin D metabolism and the relationship of these mechanisms to those utilized in other steroidogenic pathways but also into the role of the enzymes, especially the 1-hydroxylase, in diseases related to calcium metabolism. More broadly, we will be able to complete the picture of the mechanism of action of 1,25$(OH)_2D_3$ as a steroid hormone by developing an understanding of the control of the rate of its synthesis.

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