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Regulation of the Ferredoxin Component of Renal Hydroxylases at Transcriptional and Postranslational Levels and of the Protein Inhibitor of Cyclic AMP-dependent Kinase

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We have studied two proteins potentially involved in the regulation of the 25-OH-D-1-hydroxylase, which is located in the renal mitochondria and which is responsible for the production of the steroid hormone $1,25(OH)_2D_3$. The endogenous inhibitor of cyclic AMP-dependent protein kinase, PKI, is down regulated by $1,25(OH)_2D_3$. Having cloned and sequenced PKI cDNA, we studied its message levels and found them to be regulated by $1,25(OH)_2D_3$ tissue specifically in the kidney and in kidney cell culture. In other experiments we over expressed the ferredoxin component of the 1-hydroxylase and found it to be physically and chemically indistinguishable from those of classic steroidogenic tissues. The mRNA encoding the ferredoxin component is up-regulated by chronic vitamin D deficiency, which at the same time leads to sustained elevation in 1-hydroxylase activity; no short term effect of $1,25(OH)_2D_3$ on ferredoxin mRNA in kidney cell culture could be demonstrated. Finally, there was an association between decreased phosphorylation of ferredoxin and decreased 1-hydroxylase activity brought about by treatment of cultured kidney cells with TPA. Control of the renal signaling events involved in the production of $1,25(OH)_2D_3$ remains a fruitful area of investigation in the field of the metabolism and actions of vitamin D and its metabolites.

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INTRODUCTION

The renal mitochondrial hydroxylases, which are responsible for converting $25(OH)D_3$ into either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$, consist of three protein components. A flavoprotein which is associated with the mitochondrial membrane accepts electrons from NADPH and passes them to the 12 kDa matrix iron sulfur protein which in turn passes the electrons to cytochrome *P*450. The stereospecific hydroxylation of the steroid is accompanied by reduction of molecular oxygen to one molecule of water and one to the hydroxyl group to be incorporated into the steroid molecule. These enzymes are also known as mixed function oxidases and are similar, as far as we know, to the mitochondrial hydroxylases which produce the classical adrenal and gonadal steroidogenic hormones.

Many substances have been shown to alter the rate of the 1- and 24-hydroxylation of $25(OH)D_3$ in the kidney and in other cells and tissues. Our laboratory has shown, with primary cultures of chick kidney cells, that $1,25(OH)_2D_3$ reduces its own synthesis [1, 2], and induces that of $24,25(OH)_2D_3$. This result has been replicated in several other systems [3, 4]. Parathyroid hormone, PTH, has the opposite result, increasing the renal synthesis of $1,25(OH)_2D_3$ and decreasing that of $24,25(OH)_2D_3$. This result is mimicked by forskolin and cyclic AMP [5], suggesting a role of the cyclic AMP-mediated signal transduction pathway in the regulation of $25(OH)D_3$ metabolism. There is also strong evidence for the involvement of the protein kinase C signal transduction pathway in the regulation of the hydroxylation of $25(OH)D_3$, as the protein

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Table 1. Role of signaling pathways in the regulation of 25(OH)D_3 metabolism in the kidney

	Effect on production of	
Agent	1,25$(OH)_2D_3$	24,25$(OH)_2D_3$
1,25$(OH)_2D_3$	↓	↑
PTH	↑	↓
TPA, OAG	↓	↑

The table summarizes the effects of the indicated agents on the production of 1,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$ in primary cultures of chick kidney cells under a variety of experimental conditions. Similar effects are observed in other model systems. See references [1–4].

kinase C activators TPA and OAG decrease 1-hydroxylation and increase the 24-hydroxylation of 25$(OH)D_3$ [6]. These regulatory effects influencing the hydroxylation of 25$(OH)D_3$ are summarized in Table 1.

In this paper are summarized our recent results regarding the regulation of a modulator of the protein kinase A signaling system, protein kinase inhibitor (PKI) and studies examining the regulation of the ferredoxin component of the 1-hydroxylase.

THE INHIBITOR PROTEIN OF CYCLIC AMP DEPENDENT PROTEIN KINASE, PKI

PKI, is an endogenous 76 amino acid protein which inhibits the catalytic subunit of cyclic AMP-dependent protein kinase. It contains a pseudosubstrate site for kinase activity in amino acids 18–22, GRRNA, where the alanine replaces the serine which would be phosphorylated in a substrate protein. PKI not only inhibits the free catalytic subunit of cyclic AMP dependent protein kinase, but has also recently been implicated in the subcellular localization of the enzyme [7].

We showed a number of years ago [8] that the levels of the inhibitor protein are regulated by vitamin D status in the chick kidney, but not in other tissues examined thus far. PKI activity is also down-regulated by 1,25$(OH)_2D_3$ in primary cultures of chick kidney cells [9]. More recently we have found that PKI mRNA levels are up-regulated by vitamin D deficiency *in vivo* and by 1,25$(OH)_2D_3$ in cell culture [10]. We have also obtained the gene for PKI from a chick genomic library and have found that the N-terminal amino acids 1–50 are encoded by one exon and amino acids 51–76 are encoded by a second exon [11]. This is an interesting result, since the amino acids responsible for the ability of PKI to inhibit the catalytic subunit of cyclic AMP-dependent protein kinase lies entirely within the first exon. It is tempting to speculate that the portion of the peptide responsible for affecting the subcellular localization of the catalytic subunit is, at least, partially, encoded by the second exon.

We believe that the PKI gene will be an interesting system in which to study the negative regulation of gene expression by 1,25$(OH)D_3$. Furthermore, it provides a point of interaction between this steroid hormone and the cyclic AMP intracellular signaling system. There has been circumstantial evidence for such an interaction in the literature for years, and the regulation of PKI by 1,25$(OH)_2D_3$ may prove to be a key element of this interaction.

THE FERREDOXIN COMPONENT OF THE 1-HYDROXYLASE

Although it would be of great interest to study the molecular regulation of the cytochrome *P*450 component of the 1-hydroxylase, reliable reagents in the form of antibodies and cDNA probes are not yet available. In the meantime, we have focused our attention on the ferredoxin component of the enzymes which hydroxylate 25$(OH)D_3$ as a possible regulatory site in this process. In this regard, we have asked three questions, and the remainder of this paper is devoted to the answers to these questions. They are:

(1) Does chick kidney ferredoxin differ in its physical and biological properties from similar iron sulfur proteins which have been studied in the classical adrenal and gonadal steroidogenic tissues?
(2) Is the steady state level of the mRNA for ferredoxin regulated by vitamin D status or by 1,25$(OH)_2D_3$ treatment of kidney cell cultures?
(3) Is there evidence for the postranslational modification of ferredoxin which is related to the rate of 25$(OH)D_3$ 1-hydroxylation?

Properties of overexpressed chick kidney ferredoxin

To address the first question, whether chick kidney ferredoxin shares physical and chemical properties with ferredoxins from more traditional steroidogenic tissues, we subcloned a chick kidney cDNA encoding the protein preceded by a factor Xa cleavage site in Qiagen's pQE9 vector which contains, in addition to an appropriate promoter and terminator, a sequence encoding six successive histidines just in front of the multiple cloning site [12].

From this overexpression system, we obtained 7–10 mg of homogeneous fusion protein from a 1 l bacterial culture. Purification of the fusion protein was accomplished in one chromatographic step, by Ni-nitrilotriacetic acid agarose chromatography. Silver stained gels of proteins eluting between 40 and 120 mM imidazole indicated that both the fusion protein (M_W 14–15 kDa) and the mature protein resulting from factor Xa cleavage (M_W 12.5 kDa), were virtually pure. The intense brown color of the protein not only made its purification relatively straightforward, it also provided the first evidence that the iron–sulfur system was assembled correctly in this bacterial expression system.

The purity of the overexpressed chick kidney ferredoxin was confirmed by its UV-visible absorption

Table 2. Biological activity of overexpressed ferredoxin in 25-OH-D_3-1-hydroxylase assay

Fdx	AR	*P*450	[^{3}H]1,25(OH)$_2$D$_3$ (pmol/10 min/nmol Fdx)
None	+	−	0
None	+	+	0
Fusion Fdx	+	+	2.4
Mature Fdx	+	+	2.9
Adx	+	+	1.4

Cytochrome *P*450 was solubilized and partially purified from vitamin D deficient chick kidney mitochondria and assayed with the components shown. The product of the reaction, [^{3}H]1,25(OH)$_2$D$_3$, was separated by HPLC and quantitated by liquid scintillation counting.

spectrum which was typical of those published for other ferredoxins and which revealed an absorbance ratio at 414 nm/276 nm of 0.88. This is higher than the ratios of 0.6 and 0.82 published for other purified vertebrate ferredoxins [13, 14] and indicates that the overexpressed chick kidney ferredoxin was highly purified by the one-step chromatographic procedure described. The identity of the overexpressed mature chick kidney ferredoxin was confirmed by N-terminal sequencing [12].

In addition to the UV-visible absorption spectrum, the electron paramagnetic spectrum was identical to those published for other vertebrate ferredoxins, providing further evidence that the iron–sulfur center was assembled correctly in the bacterial host. Furthermore, the EPR spectrum was identical in the 6-His-fusion protein and the mature (cleaved by factor Xa) protein, indicating that the N-terminal modification had no effect on the three-dimensional structure of the site of the iron–sulfur center.

Finally, we used both overexpressed fusion ferredoxin and the mature protein in a reconstitution assay of partially purified cytochrome *P*450 which catalyzes the 1-hydroxylation of 25(OH)D$_3$. As shown in Table 2, both proteins were active in supporting the production of 1,25(OH)$_2$D$_3$ in this reconstituted system and in fact, both may be somewhat more active than bovine adrenal ferredoxin.

We conclude from these experiments that chick kidney ferredoxin has the same physical and chemical characteristics as other vertebrate ferredoxins and that this overexpression system is a convenient one in which to produce the active form of a necessary component of the 25(OH)D$_3$-1-hydroxylase. These studies provide new details in our understanding of the events in the renal cell governing the hydroxylation of 25-OH-D$_3$ study of 25(OH)D$_3$-1-hydroxylase in reconstituted systems.

Regulation of ferredoxin mRNA levels in chick kidney

Three-week-old vitamin D-deficient chicks have elevated kidney steady state mRNA levels for ferredoxin relative to vitamin D replete birds of the same age. The 40% elevation in ferredoxin mRNA levels brought about by vitamin D depletion (and concomitant maximization of 1-hydroxylase activity) was relatively modest. Using various protocols to modulate ferredoxin mRNA levels by 1,25(OH)$_2$D$_3$ treatment of primary cultures of chick kidney cells, we have been unable to demonstrate such an effect. Thus we conclude that, when animals are deprived of vitamin D in the long term, ferredoxin mRNA levels are increased, through mechanisms which remain to be investigated, but that 1,25(OH)$_2$D$_3$ does not acutely regulate the levels of ferredoxin mRNA.

Regulation of the phosphorylation state of chick kidney ferredoxin

In order to determine the role of the phosphorylation state of mitochondrial proteins in the activity of the enzymes which hydroxylate 25(OH)D$_3$ in the chick kidney, we treated primary cultures of chick kidney cells with the protein kinase C activators, TPA and OAG and with PTH and forskolin. The incorporation of ^{32}P from ATP was assessed by PAGE which revealed the TPA induced decrease in the phosphorylation of a 12.5 kDa protein. We used antibodies against a synthetic peptide representing amino acids 13–25 of chick kidney ferredoxin to demonstrate that the 12.5 kDa protein was indeed ferredoxin [15].

The dephosphorylation of ferredoxin was both rapid and transient, with about 70% dephosphorylation at 5 min treatment with TPA and a return to nearly control levels by 20 min. 25(OH)D$_3$-1-hydroxylase activity was also acutely decreased by TPA treatment at 5 min, but did not quite return to control levels at 20 min (Table 3), suggesting that the acute effect of TPA on the 1-hydroxylase may involve factors in addition to the phosphorylation state of ferredoxin.

In conclusion, we have established a potential point of interaction between the 1,25(OH)$_2$D$_3$-mediated renal intracellular events and cyclic AMP-mediated events through the regulation of PKI by 1,25(OH)$_2$D$_3$.

Table 3. Effect of TPA on ferredoxin phosphorylation and 1,25(OH)$_2$D$_3$ production in cultured chick kidney cells

Time (min) in TPA	% Maximum	
	Phosphorylation state	1,25(OH)$_2$D$_3$ production
0	100	100
5	30	60
10	35	70
20	90	70

Primary cultures of chick kidney cells were treated with TPA for the indicated times prior to collection to measure ^{32}P-incorporation into immunoprecipitated ferredoxin after a 1 h incubation with radiolabelled ATP or a further 10 min incubation with [^{3}H]25(OH)D$_3$ to measure 1-hydroxylase activity.

In addition, we have presented experimental evidence for a possible role for ferredoxin phosphorylation in the acute modulation of $1,25(OH)_2D_3$ production. These observations will provide a framework for the further understanding of the interactions between these signaling systems in the renal cell.

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