FERREDOXIN OF 25-HYDROXYVITAMIN D_3-1α-HYDROXYLASE

Anatomical distribution in the chick as determined by double-antibody radioimmunoassay

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1. Introduction

The C-25 hydroxylation of vitamin D_3 by liver microsomes [1] and the subsequent C-1 hydroxylation by the kidney mitochondrial 25-hydroxyvitamin D_3-1α-hydroxylase [2,3] result in the formation of the physiologically-active metabolite of vitamin D_3 identified as 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. This metabolite is regarded the hormonal form of the vitamin responsible for calcium and phosphate homeostasis in skeletal and soft tissue metabolism [4].

In studies of the 25-hydroxyvitamin D_3-1-hydroxylase, the requirement for NADPH and the involvement of cytochrome P450 as a terminal oxidase in the production of 1,25-$(OH)_2D_3$ were established [5–8]. The reduction of the cytochrome by NADPH is mediated by two intermediate oxidation–reduction components, namely, an iron–sulfur protein (ferredoxin) and a flavoprotein [8]. In this system, the renal ferredoxin, in a manner characteristic to the hydroxylases of a number of steroidogenic tissues, mediates the transfer of electrons from a NADPH-specific flavoprotein dehydrogenase to a cytochrome P450. The purification and properties of the ferredoxin have been described [9].

Little biochemical information at the molecular level is available concerning quantitative modulations in the amount of the 1-hydroxylase during skeletal and soft tissue disorders. This communication attempts to provide preliminary answers in the form of quantitative anatomical distribution of the 1-hydroxylase ferredoxin in the vitamin D_3-deficient chick as determined by a double-antibody radioimmunoassay (RIA) of whole tissue extracts. The production of rabbit antichick ferredoxin and goat anti-rabbit γ-globulin (IgG) antisera used in the development of the RIA are also described.

Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3

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2. Materials and methods

2.1. *Purification of chick ferredoxin*

The kidney mitochondrial ferredoxin was purified to homogeneity as in [9].

2.2. *Rabbit anti-ferredoxin antisera*

Antiserum to the ferredoxin was prepared by vaccinating 6–8 lb New Zealand White rabbits with 250 μg ferredoxin emulsified 1:1 (v/v) in Freund's complete adjuvant. The adjuvant was prepared by mixing 50 ml Aquaphor (Biersdorf Inc., South Norwalk, CN), 100 ml Marcol 52 (Exxon Corp., Oak Brook, IL) and 200 mg *M. butyricum* (Difco Laboratories, Detroit, MI). Rabbits received 0.5 ml emulsified immunogen in each rear foot pad and 0.5 ml intrascapularly. After 10 days, the vaccinations were repeated as above. The rabbits were boosted by injecting into the marginal ear vein 50 μg ferredoxin in 10 mM phosphate buffer (pH 7.4). The rabbits were bled 3–5 days after booster vaccinations and sera were collected. Antibody titer was deter-

mined by the Ouchterlony double-diffusion procedure as detailed in [10].

2.3. *Goat anti-rabbit IgG antisera*

Antiserum to rabbit IgG γ-globulins was prepared in 30–50 lb goats by two intrascapular injections of 100 mg rabbit IgG purified and characterized as in [11]. The goats were bled from the jugular veins 1 week after the second injection.

2.4. ^{125}*Iodine-ferredoxin*

The ferredoxin in 10 mM Mops buffer (pH 7.4) was radiolabeled [9] using the iodine monochloride method [12] as modified [13]. The radiolabeled protein was purified from the reaction mixture by passing the mixture through a Sephadex G-100 column equilibrated with 10 mM Mops buffer (pH 7.4). Gamma radiation in column effluents was determined using a Beckman GM-100 counter equipped with a data computer.

2.5. *Microextraction of tissue*

Tissue, 100 mg in 0.4 ml 10 mM Mops buffer (pH 7.4) containing 0.1% Lubrol WX. as homogenized in a small (5 ml) ice-cold Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was transferred into a Markson 5-ml RIA tube (Markson Science Inc., Del Mar, CA), frozen in dry ice, then immediately thawed in warm water. The freezing and thawing was repeated once again. The thawed preparation was subjected to 4 × 15 s sonications at 4°C using the microtip assembly of Heat Systems model W-375 cell disruptor (Heat Systems Ultrasonics Inc., Plainview, NY). The sonicated preparation was centrifuged at 4°C for 30 min at 7000 rev./min. The supernatant was diluted 10-fold with 10 mM Mops buffer (pH 7.4) and used in all subsequent assays. It is important to emphasize for reasons of potential clinical application that as little as 5 mg tissue could be processed as described here in a single RIA tube eliminating repetitive transfers and mechanical losses. The 10-fold dilution of final supernatant will not be necessary under such circumstances. Instead, appropriate aliquots of the supernatant could be assayed directly.

2.6. *Radioimmunoassay*

To each RIA tube were added in the order given, 0.2 ml IgG-free rabbit serum albumin fraction V (10 mg/ml) in 10 mM Mops (pH 7.4) and an appropriate aliquot of Mops buffer to give 0.3 ml total vol. when either standard ferredoxin solutions or tissue extracts were included. The tubes are vigorously vortexed to coat the inner surfaces. Appropriate aliquots of standard ferredoxin solution in 10 mM Mops buffer (5–100 μl), or aliquots of 10-fold diluted tissue extracts (5–50 μl) were then added to duplicate tubes. After the addition of a fixed amount of ^{125}I-labeled ferredoxin to each tube (8000 cpm), the contents were mixed gently. Rabbit anti-ferredoxin antiserum, 0.2 ml diluted 25-fold with 10 mM Mops buffer (pH 7.4), was then added to each tube. The mixtures were incubated with constant shaking for 30 min in a water bath at 37°C. The tubes were removed from the bath and to each was added 0.2 ml goat anti-rabbit IgG anti-serum. After thorough mixing, the tubes were allowed to stand in the cold overnight then centrifuged at 4°C for 15 min at 8000 rev./min. The supernatants were quantitatively transferred into new tubes and counted for gamma radiation. The pellets were also counted. Quantitative recovery of added radioactivity was obtained in the supernatant when all antisera were deleted from the assay incubations. A non-specific binding of 400 cpm was obtained when either antiserum was deleted. This number was used for calculating % binding of radiolabeled ferredoxin.

3. Results

The radioiodination of the ferredoxin gave spec. radioact. 0.07 μCi/μg ferredoxin. Based on this activity, the fixed amount of radioactivity added to each assay (8000 cpm) represents ~50 ng ferredoxin total. Under these conditions as little as 25 ng ferredoxin could be detected in the aliquots of tissue extracts added to assay tubes. Considering mol. wt 53 000 for the ferredoxin [9], its detectable concentration in the assay mixture would be in the range 10^{-10}–10^{-9} M.

Figure 1 shows the results of Ouchterlony double-diffusion test for rabbit anti-ferredoxin antiserum titer. The single fusing precipitin arcs seen in pattern (b) are indicative of homogeneity of the antigen (kidney ferredoxin) and of the antibodies raised to this antigen.

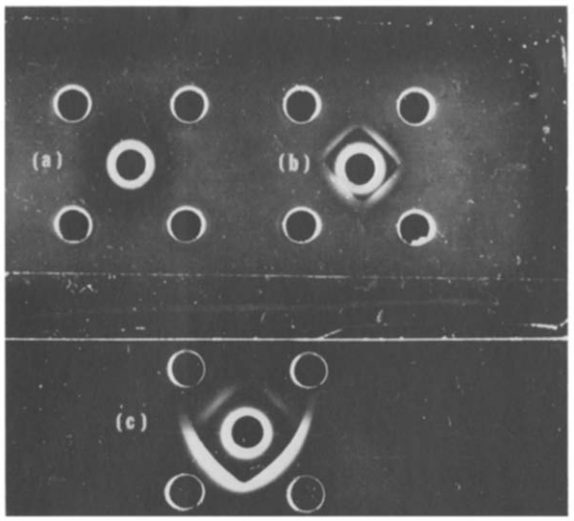

Fig.1. Ouchterlony immunodiffusion pattern. The center well in pattern (b) contained 10 μl rabbit antiserum to the chick renal mitochondrial ferredoxin. The outer wells contained renal ferredoxin beginning with upper left clockwise 1.7 μg, 3.5 μg, 5.2 μg and 7.0 μg, respectively. The pattern in (a) is the same as the pattern in (b) but the well in the center of this pattern contained 10 μl rabbit pre-immune serum. The center well of pattern (c) contained 1 mg of immunoglobulin G protein obtained from fractionated goat anti-bovine adrenal ferredoxin antisera. The outer wells contained 8.7 μg renal ferredoxin (upper left), 17.4 μg renal ferredoxin (upper right), 3.6 μg adrenal ferredoxin (lower left), and 1.5 μg adrenal ferredoxin (lower right).

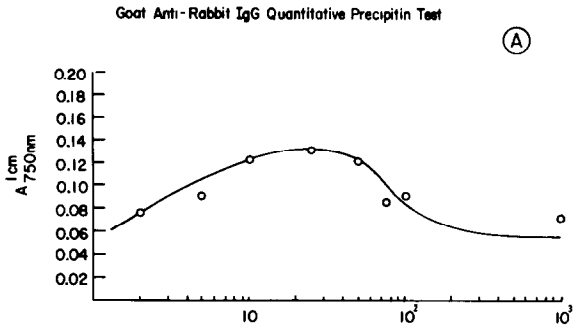

Fig.2A. Goat anti-rabbit IgG quantitative precipitin test. 1.5 ml serially diluted normal rabbit serum was mixed with 1.5 ml goat anti-rabbit IgG antiserum and incubated for 30 min in a shaking water bath at 37°C. Turbidity measurements were made spectrophotometrically at 750 nm.

Results of the quantitative precipitin test are shown in fig.2A. The formation of a complex between the rabbit γ-globulins and the goat anti-rabbit γ-globulin antiserum was maximum at 25-fold dilution of rabbit serum when both reactants were present at a ratio of 1:1. The formation of this complex was indicated as turbidity which was monitored by measuring A_{750} in an AMINCO DW-2 UV/VIS spectrophotometer. This serum dilution and ratio were, therefore, chosen for the development of the radioimmunoassay. Figure 2B shows the standard curve obtained when the % bound ^{125}I-labeled ferredoxin was plotted against known concentrations of competing unlabeled purified ferredoxin. Duplicate determinations are plotted for each concentration of ferredoxin. It can be seen that the usable sensitivity of the assay is as little as 25 ng (0.47 pmol) competing ligand. Half-maximal displacement of ^{125}I-labeled ferredoxin is obtained at 300 ng nonradioactive ferredoxin in the 0.7 ml assay volume. This corresponds to a concentration of 8 nM. It is important to note that although 10-fold diluted extracts (0.01% in Lubrol) were routinely used in the assays to give a final Lubrol concentration of 0.0007%with the use of the largest aliquot of extract (50 μl), no measurable effect of this detergent on the binding properties of ferredoxin could be detected. In fact, as much as 50 μl 0.1%

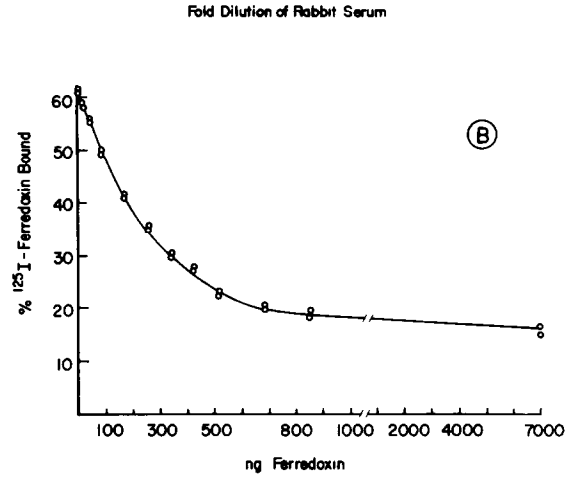

Fig.2B. Standard curve for the competitive radioimmunoassay of renal ferredoxin. See text for details.

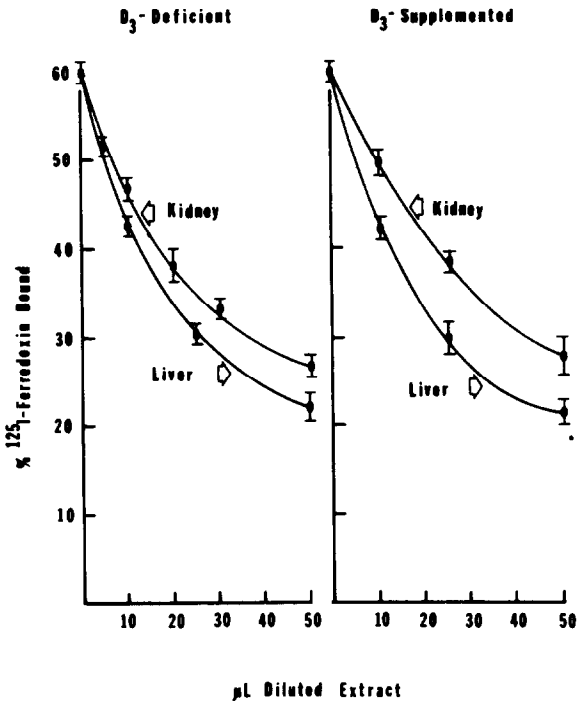

Fig.3. Comparative ferredoxin concentrations in liver and kidney of vitamin D_3-deficient versus vitamin D_3-supplemented chicks as determined by radioimmunoassay.

Lubrol (0.007% final conc.) could be used without difficulty. The variations in the values obtained from duplicate assays or from different kidneys have consistently been within $< 5\%$.

Comparative ferredoxin concentrations in liver and kidney of vitamin D_3-deficient versus vitamin D_3-supplemented chicks are shown in fig.3. First, the liver contains ferredoxin (at least a type recognized by the rabbit antibodies) at concentrations which, probably, are higher than that found in kidney. The presence of ferredoxins in liver mitochondria has only recently been documented although their quantitation has not yet been reported (14-16). Second, liver and kidney concentrations of ferredoxin do not seem to be influenced by the vitamin D_3 status of the birds. This finding is in agreement with the recent results of immunofluorescent studies which demonstrated comparable fluorescence intensities in specific parts along the nephron of vitamin D_3-deficient and vitamin D_3-sufficient kidneys (Ghazarian and Garancis, submitted for publication). It is likely, therefore, that the concentration of the renal ferredoxin is not a major factor in the modulation of the 1α-hydroxylase activity.

Table 1
Tissue distribution of chick ferredoxin

Tissue	Ferredoxin content			
	D_3-deficient		D_3-supplemented	
	µg/mg tissue	pmol/mg tissue	µg/mg tissue	pmol/mg tissue
Liver	0.64	12.0	0.64	12.0
Kidney	0.44	8.3	0.40	7.6
Testes	0.29	5.4	–[a]	–
Duodenum	0.22	4.2	–	–
Lung	0.14	2.7	–	–
Heart	0.09	1.7	–	–
Colon	0.09	1.7	–	–
Muscle	0.00	0.0	–	–

[a] Not determined

Tissue distribution of ferredoxin in the chick is shown in table 1. The most significant concentrations are those of the liver, kidney, testes and duodenum. The concentrations found in lung, heart, colon and muscle are strikingly small. This distribution appears to be consistent with the known functional significance of these tissues in hydroxylating (a) cholecalciferols (17,18), (b) testicular androgenic hormones (19), and (c) bile acids (14). The presence of ferredoxin and cytochrome P-450 in kidneys, testes, liver and intestine, or the participation of these molecules in the above biochemical events are well documented (8, 19-21).

4. Discussion

Although the 25-hydroxyvitamin D_3-1-hydroxylase appears to be exclusive to kidney tissue (16), it is possible for the ferredoxin component of this enzyme to be immunogenically similar but functionally dissimilar to ferredoxin-type proteins in other tissues. The immunogenic similarity of the beef adrenal and chick renal ferredoxins in spite of their distinctly different biofunctional roles is a clear example (fig.1

pattern c) (9, 22). While the exact relationship of the renal 1α-hydroxylase ferredoxin to those of non-renal tissue is not known, reports of the occurrence of cytochrome P-450 and hydroxylating reactions, probably requiring ferredoxins, in duodenum and other non-renal tissues (16-22) underscore the significance of iron-sulfur proteins.

In the chick, as in man, the kidney progresses through 3 stages of development; pronephros, mesonephros and metanephros. The latter is the definitive kidney. However, unlike the mammalian kidney, the unambiguous disection of the amorphous chick kidney to distinct medullary and cortical regions is not possible. In our continuing RIA studies with disected beef and rabbit kidney medullary and cortical tissues, the association of the ferredoxin with the juxtaglomerular apparatus (JGA) was suggested (J.G.G., Garancis, submitted). The relevance of this is that the JGA plays an important role in autoregulation of renal blood flow and in glomerular filtration rate. This regulation is mediated through the elaboration of renin by the juxtaglomerular cells in the wall of the afferent arteriole proximal to the glomerulus. The JGA is in an ideal position to 'monitor' incoming circulation since this area of the kidney receives the greatest blood flow. Because 25-OH-D_3 is converted in the kidney to the active hormone, 1,25-$(OH)_2D_3$, and the production of the hormone is rigorously controlled by circulating blood calcium and phosphate levels, it would be reasonable to assume that the site of 1-hydroxylation in the kidney is in a position well exposed to ample blood flow. Such a position, already assigned one endocrine function (renin production), could be the juxtaglomerular apparatus.

Finally, while bioassays and receptor binding assays are available [23] for the assessment of 1-hydroxylase activity, their disadvantages are that only the product of the enzyme activity is the parameter measured and not the actual concentration of enzyme molecules. On the other hand, RIA directly measures modulations in enzyme concentration independent of activity. This property is of paramount importance in the potential application of the procedures we have described to the quantitative evaluation of pathochemical changes in skeletal and soft tissue disorders that may involve the 25-hydroxyvitamin D_3-1-hydroxylase.

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