AFFINITY DIFFERENCES FOR THE 25-OH-D_3 ASSOCIATED WITH THE GENETIC HETEROGENEITY OF THE VITAMIN D-BINDING PROTEIN

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1. Introduction

Vitamin D, exogenous or endogenous, must be transformed to an hydroxylated metabolite (e.g., 25-OH-D_3, 24,25-OH-D_3) to become an active metabolite of the bone tissues or to participate in the regulation of phosphocalcic metabolism [1–4]. This hydroxylation must necessarily be performed in the liver and in the kidneys. The various stages from the synthesis or ingestion of vitamin D to its arrival and metabolism in the target tissues are dependent on the existence of a plasmic binding protein: the vitamin D-binding-protein (VDBP) This globulin was shown in [5] to be identical to an α_2-globulin already known as group-specific component (Gc). The heterogeneity of this protein is due to a large genetic polymorphism [6] (3 genes Gc^{1F}, Gc^{1S}, Gc^2 and 26 known variants). Here the gene nomenclature is Gc^{1F}, Gc^{1S} or Gc^2 with the corresponding Gc 1F-1F, Gc 1S-1S, or Gc 2-2 for the homozygous phenotypes and Gc 2-1F, Gc 2-1S or Gc 1F-1S for the heterozygous phenotypes. After electrophoresis, the Gc^2 gene product was observed as a single band (the Gc 2 protein) while the Gc^{1F} or Gc^{1S} gene products (the Gc 1F or Gc 1S proteins) were each characterized by a set of two bands: Gc 1a for the anodal band; Gc 1c for the cathodal band. The presence in the serum of the Gc 1a protein band was considered as the post-transcriptional transformation of the Gc 1c protein in relation to its metabolism [7,8].

Thus it seemed interesting to investigate the relations between the genetic differences responsible for its heterogeneity and the affinity of the proteins for the 25-OH-D_3. Usually any investigation in this field requires the preliminary step of the preparation of a highly purified protein. The main difficulties encountered in these procedures are related to the low concentration in the serum (~0.3 mg/ml), the time required and the cost if a good quality protein is to be obtained [3,9,10], and the biological properties of the protein must also be preserved. The methods were poorly adapted to the research we have undertaken. For these reasons we chose the analytical electrophoresis procedures such as two dimensional electrophoresis on PAGE (polyacrylamide gel electrophoresis) and IEF (isoelectrofocussing).

2. Materials and methods

2.1. *Electrophoretic techniques*

Electrophoresis on polyacrylamide gel was as in [11] using a system of discontinuous buffers (Tris, EDTA, boric acid), a running buffer (pH 8.28) and a gel buffer (pH 8.91). This technique was the most appropriate to separate the protein bands of the VDBP from albumin and other globulins. The vertical electrophoresis was on acrylamide plates 13 × 8 × 0.3 cm (Pharmacia GE 4). The spacer gel was acrylamide 2.5%, bis-acrylamide 0.62% (Eastman Kodak) and the migration gel was 6.6% acrylamide and 0.2% bis-acrylamide. Both gels were polymerised in the presence of Temed and ammonium persulfate (Merck). The migration lasted 3 h at 10°C and 15 V/cm.

IEF was done on a polyacrylamide gel (4.85% acrylamide, 0.15% bis-acrylamide), 1 mm thick (Multiphor LKB). The gel contained an ampholyte solution (pH 4.6) (Ampholines LKB) 2% and a riboflavine solution which allowed photopolymerisation. The conditions of the electrophoresis were: 1200 V max; 15 W max; 20 mA max; for 4.5 h. The temperature was maintained at 10°C. Strips of Whatman 17 were soaked with 1 M H_3PO_4 at the anode and 0.2 M NaOH at the cathode. The serum samples were applied on the gel 1 cm from the cathode with Whatman paper 3MM.

2.2. *Two-dimensional electrophoresis*

The first dimension was performed on PAGE, the second on IEF. After the first dimension, using as reference the position of serum albumin, a band of 4 × 0.5 cm was cut out from the gel, and placed on the surface of the IEF gel 1 cm from the cathode (fig.1).

2.3. *Immunofixation procedure*

After the IEF, the VDBP electrophoretic pattern could only be detected by a specific method such as print-immunofixation [12] using a monospecific antiserum anti Gc (Dakopatts). The cellulose-acetate bands were stained with Coomassie blue solution (R-250).

2.4. *pH measurements*

In these experiments the pH values were obtained with an Ingold surface electrode (type lot 403/30) at the migration temperature across the surface of the gel from the anode to the cathode. At least 3 measurements were made to estimate a mean value for the pH [13].

2.5. *Test for saturation of the VDBP by 25-OH-D_3*

The solutions were prepared by successive dilutions of an ethanol solution of 25-OH-D_3. The amount theoretically necessary to obtain the saturation of the apoprotein form of the VDBP was calculated on the basis of the protein concentration and to a mole to mole reaction with the ligand. The affinity constant obtained by the Scatchard method is high for this vitamin D_3 derivative: K_a 1–2 × 10^8 M^{-1} [11,12].

3. Results and discussion

When increasing concentrations of 25-OH-D_3 were added to a serum there was a progressive saturation of the protein and a transformation of the free form (apoprotein) to the bound form (holoprotein). These two forms could be separated by PAGE, the holoprotein having a more anodic mobility than the apoprotein [14,15]. Fig.1 shows the two-dimensional electrophoretic patterns obtained with a serum phenotype Gc 2-1 (i.e., Gc 2-1F or Gc 2-1S) in the presence

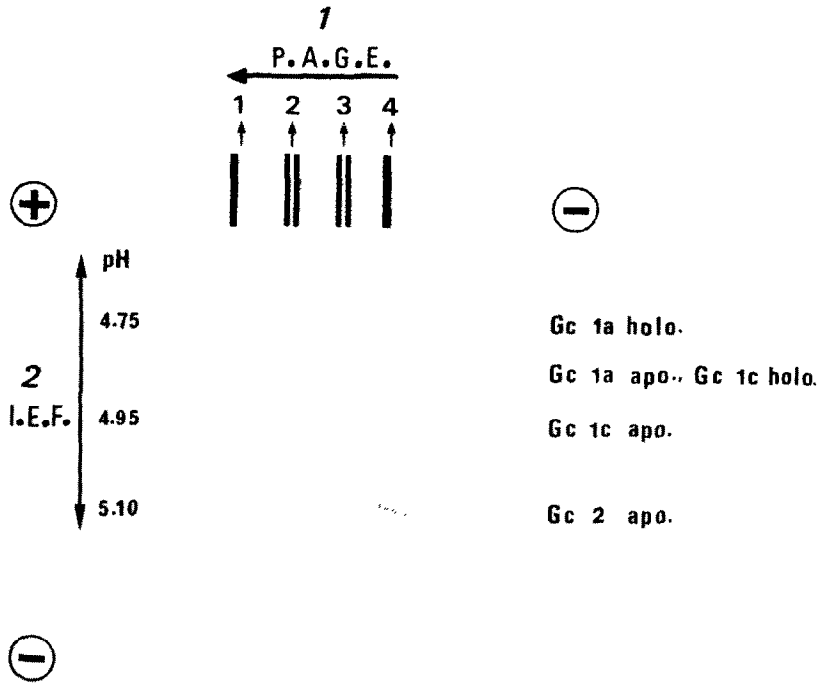

Fig.1. Two-dimensional electrophoretic pattern of a Gc 2-1 sample partially saturated with 25-OH-D_3 (1 μg/ml serum). The band positions after the t dimension (PAGE) are: (arrow 1) Gc 1a holoprotein; (arrow 2) Gc 1a apoprotein, Gc 1c holoprotein; (arrow 3) Gc 1c apoprotein, Gc 2 holoprotein; (arrow 4) Gc 2 apoprotein. Each holoprotein form presents an anodic mobility while the excess of the protein bands in the apoprotein form shows a cathodic mobility. During the second dimension (IEF) the protein–ligand exchange takes place to the detriment of the Gc 2 holoprotein.

of a non-saturating dose of 25-OH-D_3. After the first dimension in PAGE (running buffer, pH 8.28) 6 different bands were expected [3,14]. Based on [7,9] using homozygous phenotypes, the proteins corresponding to those bands were determined. The most anodic was the Gc 1a holoform (arrow 1), then a set of 2 bands hardly separated and corresponding to the Gc 1a apoprotein and the Gc 1c holoprotein (arrow 2). Another set of 2 bands (arrow 3) was composed of Gc 2 holoprotein and Gc 1c apoprotein. The last and sixth band is represented by the Gc 2 apoprotein (arrow 4). Under these conditions, there is no affinity difference of any protein band for the 25-OH-D_3. This is in agreement with [16]. During the second dimension (IEF) an extremely fast exchange occured between the different apoprotein and holoprotein bands. After IEF the Gc 1a holoprotein remained as a single band (fig.1). Corresponding to arrow 2 we observed the presence of the Gc 1a apoprotein and of the Gc 1c holoprotein, but also of the Gc 1a holoprotein and the Gc 1c apoprotein; after the first dimension the Gc 1a holoprotein could not be present in this position. It was generated during the second dimension to the detriment of the Gc 1c holoprotein by the following mechanism:

> Part of the Gc 1a apoprotein picks up the 25-OH-D_3 of the Gc 1c holoprotein and moves to the position of the Gc 1a holoprotein giving thereby rise to the Gc 1c apoprotein.

An identical exchange (Gc 1c apoprotein and Gc 2 holoprotein together after PAGE, arrow (3) takes place to the detriment of the Gc 2 band. In these conditions, the Gc 2 holoprotein band whose position would be between the Gc 1c apoprotein and the Gc 1a apoprotein, has completely disappeared. These exchanges cannot affect the Gc 1a holoprotein and the Gc 2 apoprotein bands separated in the first dimension. They remained unchanged during the second dimension (IEF).

These results show under IEF conditions the preference of the ligand for the Gc 1a band and later on for the Gc 2 band. To confirm this, an experiment was performed to study directly the exchange between the Gc 2 holoprotein and the different Gc 1S apoprotein bands. In fig.2(1) (right sample) the Gc 1S apoprotein sample was applied over 3 mm. After a pre-focussing migration step, the Gc 2 holoprotein sample was applied (fig.2(2)) at the same place as the previous sample but over 10 mm. This sample could be represented as divided into 3 parts: (a,c) represent the Gc 2 holoprotein not submitted to the exchange with the Gc 1S bands and focussed at its isoelectric point (4.90–4.95); in (b) the Gc 2 holoprotein first moves to its isoelectric point (arrow 1) where the Gc 1c apoprotein band has focussed. Then when the two bands were together, the Gc 1c band carried off the 25-OH-D_3 on the Gc 2 holoprotein. The Gc 1c apoprotein was transformed into its holoform while the Gc 2 holoprotein returned to its apoprotein form and migrated back to the pH 5.10 zone corresponding to its new pI (arrow 5). The Gc 1c holoprotein migrated towards its pI (arrow 2) and when it reached the Gc 1a apoprotein band a similar exchange occurred between the Gc 1a apoprotein and the Gc 1c holoprotein. The Gc 1a apoprotein was transformed into the holoform and migrated towards the anode (arrow 3; pI 4.75), while the resulting Gc 1c apoprotein returned to its isoelectric point (arrow 4). Fig.2(3) shows the result obtained after such exchanges of 25-OH-D_3 between the Gc 1 and Gc 2 proteins. The isoelectric-focusing pattern of the Gc 1S-1F proteins is presented as reference on the left of each diagram in fig.2. We were thus able to see that the protein–ligand complex as studied after IEF does not correspond to denaturation. The ligand transported by the holoprotein remained permanently exchangeable. These reactions of binding and dissociation took place under conditions of pH close to the isoelectric points.

The results obtained in a third experiment are shown in fig.3. In the presence of increasing concentrations of 25-OH-D_3 added to a Gc 1F-1S serum, the Gc 1a holoprotein produced by the Gc^{1F} gene presented first the anodic shift corresponding to the appearance of the Gc 1a holoprotein form (sample 3), and until this band was transformed into its holoprotein form, the homologous protein synthesized by the Gc^{1S} gene was not affected (sample 4). The anodic shift of the Gc 1a protein was complete in sample 5 (0.5 μg 25-OH-D_3/ml serum). From sample 6–11 it is possible to follow the different steps of the anodic shift of the Gc 1c proteins. The Gc 1c protein produced by the Gc^{1S} gene is the last to present the anodic shift.

These exchanges of the ligand between the holoprotein and apoprotein bands as observed in fig.1,2 are not an artefact due to the IEF conditions but correspond to affinity differences between the proteins for the ligand. These affinities seem to be thus closely linked to the different pI of the protein bands. The lower the pI of the protein the greater the affinity

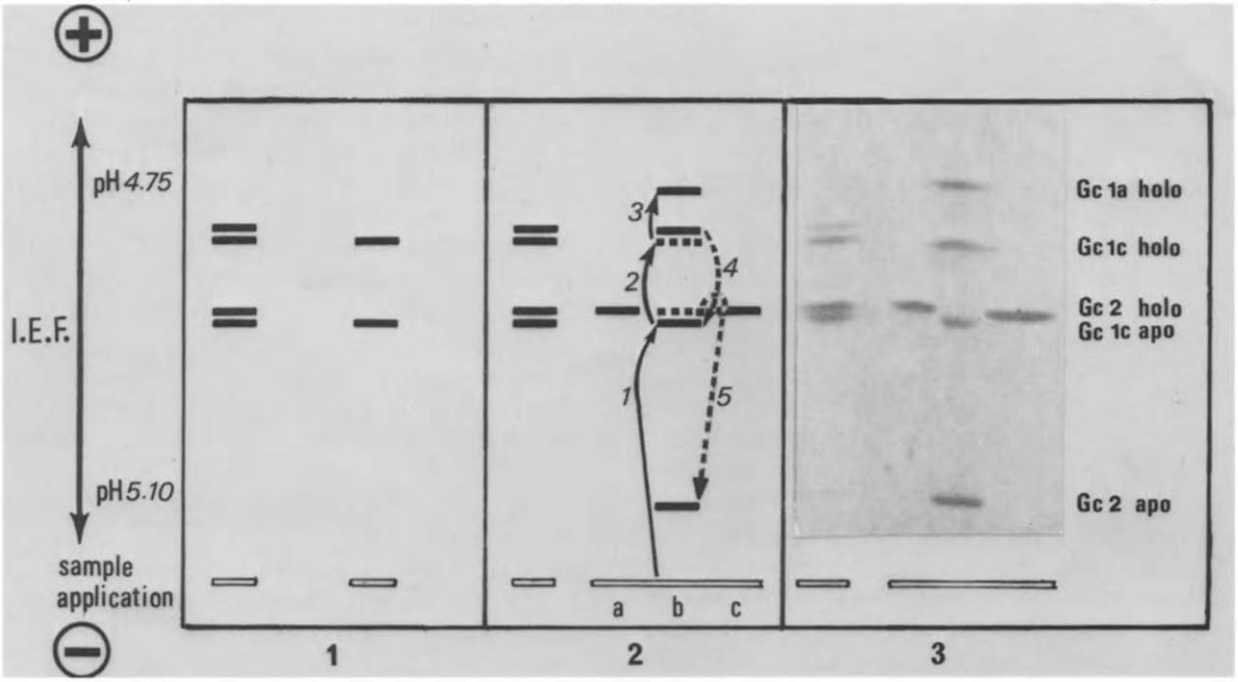

Fig.2. The 2 schematic representations of this figure outline the steps of the experiment performed to show that the ligand remains exchangeable between the proteins with different affinities. The 4 bands located on the left of each of the 3 parts of the figure correspond to the electrophoretic pattern obtained with a Gc 1F-1S serum sample used as reference for the mobilities of the apoproteins. (1) First a sample of Gc 1S-1S is applied on the cathodic side of the gel with a small (3 × 0.5 mm) Whatman 3MM paper strip. After migration (1 h), the Gc bands are focussed, giving the Gc 1Sc apoprotein on the cathodic side, and the Gc 1Sa apoprotein on the anodic side. (2) This diagram shows the second step of the experiment. After accomplishing the first step, a sample of the Gc 2 holoform is applied on the same position as was applied the Gc 1S-1S sample (diagram 1) but with a longer (10 × 0.5 mm) Whatman 3MM paper strip. A second IEF is then performed for 3 h. During this time the ligand carried by the Gc 2 holoprotein is exchanged between the different Gc 1a and Gc 1c apoproteins (arrows 1–3) as explained in the text. The dotted arrows 4,5 show the cathodic shifts of the holoproteins after exchange of the ligand. (3) This shows the final result obtained after ligand exchange between holoproteins and apoproteins as presented in diagrams (1,2).

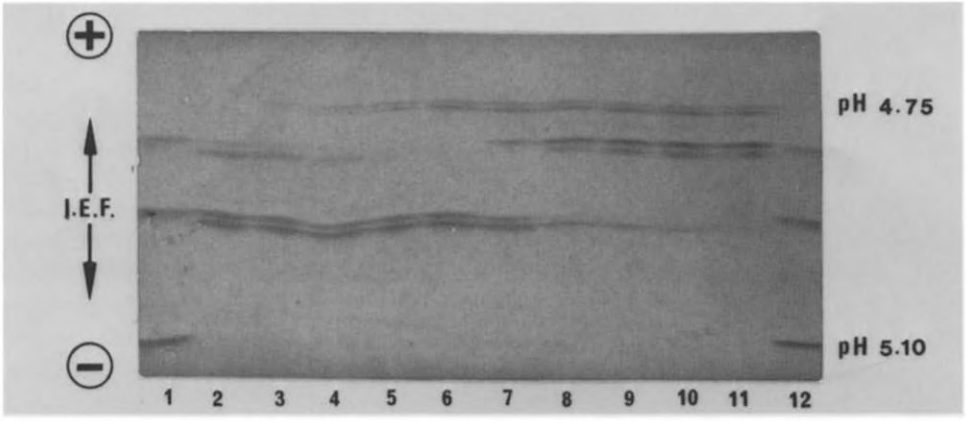

Fig.3. Here a Gc 1F-1S serum is used to demonstrate the importance of the pI differences between the proteins in their affinities for the ligand. A progressive saturation with the 25-OH-D_3 solution is obtained. In samples 1,12, the 3 bands obtained after IEF correspond to a Gc 2-1F serum. The most cathodic band corresponds to the Gc 2 protein. In the anodic zone the set of 2 bands corresponds to the Gc 1F protein. In sample 2 the electrophoretic pattern obtained corresponds to Gc 1F-1S serum. Four bands are observed. The Gc 1Sc and Gc 1Fc protein bands are the more cathodic bands while the Gc 1Fa and Gc 1Sa protein bands are the more anodic bands; samples 3–11 show the results of the progressive addition of 25-OH-D_3 (0.25, 0.33, 0.5, 0.7, 1, 1.4, 1.8, 2 and 3 μg 25-OH-D_3/ml serum, respectively) to the Gc 1F-1S sample.

of the protein for its ligand. These protein activities which are extremely sensitive can only be demonstrated under IEF, i.e., when the proteins are at a pH which is almost identical to their isoelectric point and not in a buffered system as with PAGE.

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