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ISOELECTRIC FOCUSING OF THE PHOSPHOPROTEIN OF RAT-INCISOR DENTIN IN AMPHOLINE AND ACID pH GRADIENTS

EVIDENCE FOR CARRIER AMPHOLYTE–PROTEIN COMPLEXES

MATS JONSSON and STIG FREDRIKSSON

Department of Physical Chemistry, Chalmers University of Technology and University of Göteborg, S-402 20 Göteborg 5 (Sweden)

and

MATS JONTELL and ANDERS LINDE

Laboratory of Oral Biology, Department of Histology, University of Göteborg, S-400 33 Göteborg 33 (Sweden)

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SUMMARY

Rat-incisor phosphoprotein (RIP) has been subjected to isoelectric focusing at 4° in (a) an Ampholine pH gradient of 2.5–4 and (b) an acid pH gradient created by electrolysis of a system of acids and acidic ampholytes and covering the pH range 0.5–3.5. In the Ampholine gradient, the RIP unexpectedly formed several adjacent and strongly opalescent bands in the pH range 2.5–3.1. These bands, which migrated slowly toward the anode on prolonged focusing, are interpreted as being the result of an interaction between the amino groups of the Ampholine and the numerous phosphate groups of the protein. In the acid pH gradient, the RIP focused into one narrow zone corresponding to an isoelectric pH of 1.1 at 4°. This value is consistent with the amino-acid composition and the phosphate content of the protein.

INTRODUCTION

Besides collagen, preparations of decalcified dentin of different species have proved to contain a class of proteins rich in aspartic acid, serine and organically bound phosphate[1–5]. These phosphoproteins are of special interest to workers in the field of calcified tissues, as it has been postulated[1,6] that they play an essential role in the mechanism by which hydroxyapatite crystals are deposited within the organic matrix.

However, from a methodological point of view, the dentin phosphoproteins are also very interesting to those working in the field of isoelectric focusing (IEF). Because of their high content of strongly acid phosphate groups, but low content of basic groups, these proteins would be expected to have isoelectric points too low

to permit focusing within the usual carrier-ampholyte pH gradients (which cover pH values down to about 2.5). For the phosphoprotein of rat incisors (RIP), for instance, a rough estimate, made by means of the Linderstrøm–Lang equation[7,8] and based on the amino acid and phosphate contents[5], indicates a p*I* value of *ca.* 1. Running the RIP in a pH gradient established from commercial carrier ampholytes would thus be of little value, as the protein should focus near the anode, where accurate evaluation of its p*I* is impossible owing to the steepness of the pH course.

Surprisingly, this conclusion has proved to be erroneous. If the RIP is subjected to IEF in an Ampholine pH gradient of 2.5–4 created in the 1.5-ml column described earlier[8,9], the final 280-nm scan shows a complicated pattern with several adjacent peaks in the pH region 2.5–3.1.

On the other hand, the RIP focuses into one narrow zone in the expected pH region if the Ampholine pH gradient is exchanged for one created by electrolysis of a system of acids and acidic ampholytes and covering the pH range 0.5–3.5.

EXPERIMENTAL

Isolation and purification of RIP

Incisors dissected from Sprague–Dawley rats were freed from adhering soft tissue, dried and disaggregated. RIP was isolated either by demineralization of the teeth in 0.5 *M* acetic acid and subsequent extraction with 1 *M* NaCl in 50 m*M* Tris–hydrochloric acid buffer[2] (henceforth referred to as HAc-RIP) or by simultaneous demineralization and extraction with neutral 0.5 *M* EDTA[10] (EDTA-RIP). After dialysis against distilled water and freeze-drying, the crude RIP preparations were purified by molecular-sieve chromatography on Sepharose 4B, followed by ion-exchange chromatography on DEAE-cellulose[5].

IEF in Ampholine pH gradient

The 1.5-ml column described earlier[8] was filled with the following system of aqueous solutions: 0.30 ml of anode solution (containing 0.1 μl of concentrated sulphuric acid per ml of water), 0.20 ml of top solution (1% of Ampholine pH 2.5–4), 0.70 ml of intermediate solution (1% of Ampholine pH 2.5–4, 120 g/l of sucrose and 30–60 μg of RIP), 0.30 ml of bottom solution (1% of Ampholine pH 2.5–4 and 200 g/l of sucrose) and 0.23 ml of cathode solution (0.75% of Ampholine pH 2.5–4, 0.25% of Ampholine pH 5–7 and 200 g/l of sucrose). Formation of the density gradient, scanning of the column during the run, fractionation of the column contents after completion of the run and subsequent evaluation of the pH course were carried out as described previously[8,9,11].

IEF in acid pH gradient

Ampholine-free pH gradients covering the approximate pH range 0.5–3.5 were created in an LKB 110-ml column (Type 8100-1) by the method developed by Jonsson and Pettersson[12,13]. The dense and less-dense solutions used for the formation of the density gradient were composed as follows. In addition to the sample (8–20 mg of RIP), the dense solution consisted of 0.30 ml of concentrated sulphuric acid ($pK_1 \approx -3$, pK_2 1.99 at 25°) and 30 g of sorbitol in 60 ml of water. The less-dense solution contained 0.30 g of citric acid (pK_1 3.08), 0.30 ml of concentrated formic acid (p*K*

3.75), 0.20 ml of concentrated acetic acid (pK 4.75), 0.10 g of aspartic acid (pI 2.98 and 0.15 g of glutamic acid (pI 3.22) in 55 ml of water.

The anode was at the bottom of the column, 0.085 M sulphuric acid being used as the anode solution and 0.025 M sodium hydroxide as the cathode solution. The bottom electrode solution also contained 530 g/l of sorbitol.

The procedure used for filling the column differed from that recommended by the manufacturer. The bottom electrode solution (*ca.* 11 ml) was slowly pumped into the column *after* the density gradient had been introduced through the top nipple in the usual way. This modification minimized the risk of a skew distribution of the bottom electrolyte, with the ensuing formation of a slanting protein zone on focusing[14].

Before layering 5 ml of cathode solution on top of the density gradient, the upper 10 ml of the latter were exchanged for 10 ml of a solution of histidine (0.45 g of histidine per 15 ml of water). As demonstrated by Pettersson[13], histidine (pI 7.47) prevents quenching of the current caused by the formation of an electrolyte-free region between the weakest acid and the cathode base.

The column was thermostatically controlled at 4.0°, and constant power of *ca.* 0.8 W was applied. As the focusing was slow in an acid pH gradient (due to the high conductance), electrolysis was continued for *ca.* 160 h.

After the runs were finished, the column contents were fractionated into 2-ml fractions, with one exception. When focusing samples of HAc-RIP and EDTA-RIP simultaneously, that part of the column contents expected to contain protein was divided into 0.7-ml fractions in order to increase the resolution of the protein assay (see below).

Evaluation of acid pH gradient

The pH values of the 2-ml fractions were measured at 4.0 and 25.0° by means of the equipment described elsewhere[15]; those of the 0.7-ml fractions were measured at 25.0° as described by Fredriksson[11]. The electrodes were standardized with commercial buffers (Radiometer S1510 and S1316; Merck, pH 1.00).

Protein assay

As RIP has a low absorptivity at 280 nm[5] and, moreover, the sorbitol is partly degraded into strongly UV-absorbing substances in the acid pH gradient[13], the RIP content of the fractions was monitored by two other techniques.

(*i*) After dialysis of 100 μl of each fraction against distilled water, the relative phosphate content was determined by a modified molybdate assay[5,10].

(*ii*) Like other polyanionic biopolymers, the RIP is precipitated by long-chain aliphatic ammonium salts such as cetylpyridinium chloride (CPC). Thus, the relative amount of RIP in each fraction could be estimated simply by adding CPC and measuring the resulting turbidity at 550 nm. A good correlation between the phosphate and CPC assays was obtained (*cf.* Fig. 2a).

Normally, 300 μl of a 1% (w/v) solution of CPC (Sigma, St. Louis, Mo., U.S.A.) were added to 100 μl of each fraction. In the experiment reported in Fig. 2c, the ratio of CPC and fraction volumes was reversed because the loading of RIP was smaller by a factor of 3.

RESULTS AND DISCUSSION

As mentioned in the introduction, the IEF of RIP in an Ampholine pH gradient 2.5–4 gave a surprising result (Fig. 1). Instead of accumulating close to the anode as expected, the protein spread over a wide pH range (2.5–3.1), showing several peaks that were not clearly separated from each other.

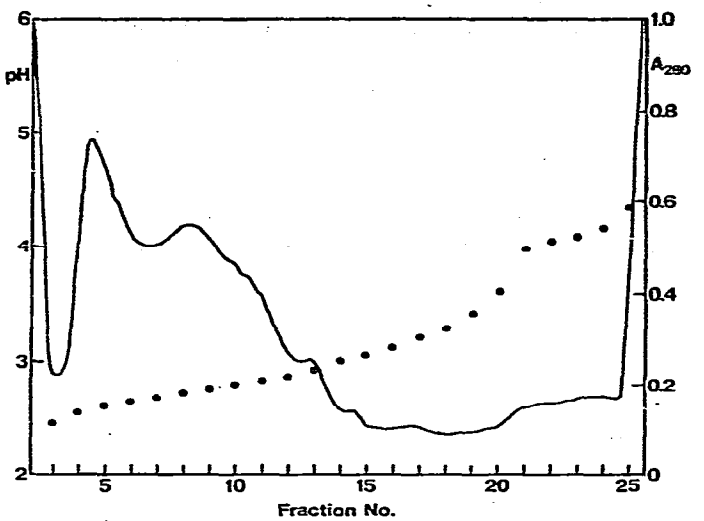

Fig. 1. Scan of 1.5-ml column[8,9] at 280 nm (solid curve) obtained after density-gradient IEF at 4° of 42 μg of HAc-RIP in 1% of Ampholine pH 2.5–4 for 105 min at an average field strength of 20 V/cm. Superimposed are pH values at 25° of 60-μl fractions of the column contents[11].

On visual inspection, the corresponding part of the column contents was found to be strongly opalescent, and with higher loadings of RIP, a precipitate was also observed*. Moreover, repeated scanning of the column revealed a slow migration and redistribution of the sample towards the anode on prolonged focusing.

These observations show that the peaks and shoulders in Fig. 1 do not represent different proteins, but are due to the formation of Ampholine–protein complexes, probably through interaction between the amino groups of the Ampholine and the numerous phosphate groups of the RIP. In fact, a recent paper by Righetti *et al.*[16] on the IEF of acidic dyes in polyacrylamide gel supports this assumption. By altering the ratio of dye to carrier ampholyte, these workers obtained evidence for the formation of strong complexes between the Ampholine and some dyes containing sulphonate groups.

Similar experiments with RIP proved unsuccessful, however, because small amounts of this protein could not be localized by staining, and larger amounts were precipitated. The affinity of Ampholine for the RIP is so high that cationic dyes such as toluidine blue and alcian blue, which bind to the protein in ordinary acidic buffers, are displaced by the carrier ampholytes. This was evident from a spectro-

* A certain opalescence was observed when the protein was added to the initial solution of Ampholine. On the other hand, an equally concentrated solution of RIP in water was perfectly clear.

photometric competitive-binding study carried out essentially as described by Righetti and Gianazza for heparin[17].

Indirect evidence of the formation of complexes between RIP and Ampholine was obtained when the Ampholine pH gradient was exchanged for an acid pH gradient created by electrolysis of a mixture of acids and acidic ampholytes. IEF of a sample of HAc- or EDTA-RIP in the latter type of pH gradient (see Figs. 2a and 2b) gave rise to only one peak in the absorbance curve at 280 nm (shown in Fig. 2a only*).

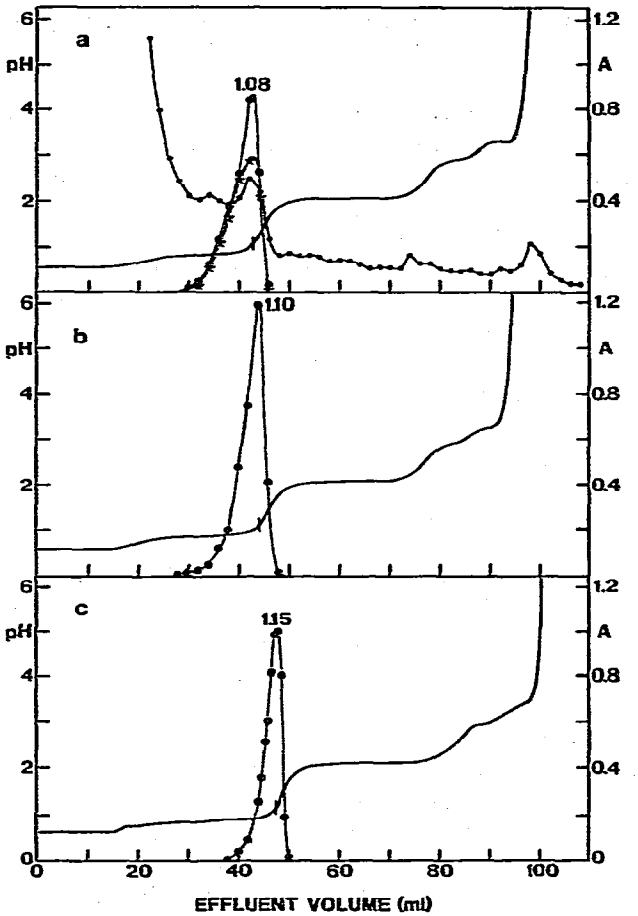

Fig. 2. Distribution of RIP obtained in an LKB 110-ml column after density-gradient IEF at 4° in a pH gradient created by electrolysis of a system of acids and acidic ampholytes[12,13]. Time of focusing 160 h; power *ca.* 0.8 W. The resulting pH course as measured at 25° (see ref. 15) is shown by the solid curve. The RIP content of 2- or 0.7-ml fractions of column effluent was monitored by means of the UV absorbance at 280 nm (•), a colorimetric phosphate assay[5,10] at 700 nm (★), and/or CPC-turbidity measurements at 550 nm (◉). The protein samples consisted of: (a) 20 mg of HAc-RIP; (b), 20 mg of EDTA-RIP and (c), 4 mg each of HAc-RIP and EDTA-RIP. The figures at the peaks are the p*I* values of the RIP samples.

* The rest of the peaks in the UV absorbance curve were present also in a run without protein.

The assays made on the fractionated effluent from the column confirmed that the corresponding zone contained RIP. Moreover, comparison of the absorbance and pH curves revealed that the protein had focused in the expected region.

In a previous communication[5], samples of RIP prepared by the acetic acid–sodium chloride and EDTA extraction procedures were compared. The two preparations displayed a number of common properties, *e.g.*, a molecular weight of 30,000 ± 2000, similar chromatographic behaviour, and identical electrophoretic mobilities in Tris–hydrochloric acid buffer (pH 8.4). On the other hand, the amino acid analyses indicated a 15% lower content of serine in EDTA-RIP. It was concluded, however, that with regard to the inherent uncertainty in the methods of analysis employed, no definite difference in composition between HAc- and EDTA-RIP could be established.

The results of the IEF runs seem to support this conclusion. EDTA-RIP (Fig. 2b) focuses at the same pH as HAc-RIP (Fig. 2a). If equal amounts of HAc-RIP and EDTA-RIP are focused simultaneously, a single non-broadened peak is obtained (Fig. 2c).

It should be remembered, however, that a pH gradient shaped as in Fig. 2 is not ideal for the disclosure of small variations in the amino acid composition of RIP preparations. Unfortunately, the p*I* of RIP is located in a region where the pH course shows a steep transition from one shallow part to another. The resolving power in this part of the pH gradient is probably not better than 0.1 unit of pH.

As demonstrated by Fredriksson[18], the pH value assigned to the concentration maximum of a focused protein will not necessarily be the true p*I* of the protein if the temperatures of focusing and pH measurement do not coincide. In an Ampholine pH gradient, for instance, the p*I* value evaluated from an IEF run at 4° followed by pH measurements at 25° may differ by as much as 0.2 unit of pH from the true value at 25°.

In order to investigate the influence of the temperature of pH measurement on the acid pH gradient, two runs with HAc-RIP were carried out at 4°, the pH course being mapped at 4° in one run and at 25° in the other; the resulting p*I* values for RIP were 1.10 and 1.08, respectively. As the disparity between these values is less than the experimental uncertainty, the pH courses of the subsequent runs were evaluated at the more convenient temperature of 25°.

With a knowledge of the isoelectric pH and the amino acid composition of RIP, and by assuming that the isoelectric pH coincides with the isoionic pH, *i.e.*, binding of anions to the protein is negligible, it is possible to estimate the intrinsic dissociation constant (K_{int}) of the phosphate groups by reversing the calculation mentioned in the introduction. From the data of Jontell *et al.*[5], the number of ionic groups per molecule of HAc-RIP can be calculated as 99 β,γ-carboxy-groups, 1 α-carboxy-group, 63 phosphate groups and 6 basic groups. If the pK_{int} values for the carboxy- and the basic groups are assumed to be normal (*i.e.*, 4.6, 3.8 and >6.5, respectively[19]), the isoelectric condition of zero net charge on the protein molecule requires the average degree of dissociation, x_i (see ref. 8), to be 6/63 for the phosphate groups at pH 1.1. Insertion of these figures into the Linderstrøm-Lang equation[8] gives

$$pK_{int} = 1.1 - \log \frac{6/63}{1 - 6/63} = 2.1$$

For EDTA-RIP, which has 66 phosphate and 9 basic groups according to the phosphate and amino acid analyses[5], analogous reasoning gives a pK_{int} value of 1.9.

Unfortunately, the pK_{int} values obtained are difficult to compare with literature data. As, to our knowledge, no experimental values have hitherto been reported for the pK_1 of orthophosphate bound in monoester linkage to a protein, we are reduced to making a comparison with small model compounds resembling protein-linked phosphate. According to Tanford[19], glycerol 2-phosphate, with a pK_1 of 1.3 at 25°, is a relevant molecule. For a number of small peptides, which (like RIP) contained phosphoserine, Fölsch and Österberg[20] obtained $pK_1 \leqslant 1$ (and pK_2 5.4–6.0) for the phosphate group. In view of these data, our values for the phosphate groups of RIP are much too high.

In itself, the discrepancy is not surprising, as we have applied the Linderstrøm-Lang equation to a protein that is probably non-globular and has its charge unevenly distributed over the surface. It is interesting to note, however, that Lee *et al.*[21], in a recent nuclear magnetic resonance study of the phosphoprotein of bovine dentin measured an average pK_2 of 6.8 for the phosphate groups. As this value is considerably higher than the pK_2 values obtained by Fölsch and Österberg, and at the same time not far from the pK_2 of orthophosphoric acid (7.2 at 25°), it may be that the phosphate groups of RIP in fact have an average pK_1 close to that of orthophosphoric acid (*i.e.*, 2.1 at 25°).

The experiments reported in this paper have opened an important field of application for acid pH gradients covering pH values below 3. So far, phosphoproteins are known to be present in the dentin of six mammalian species (rat, guinea pig, rabbit, dog, ox and man[4]. Phosphoproteins have also been isolated from egg yolk[22] and pig brain[23], and it is likely that other strongly acid phosphoproteins will be found in future.

In this situation, it is unsatisfactory that the design of an acid pH gradient of appropriate range and slope is still mostly a matter of trial and error. We have therefore initiated a systematic investigation of the formation of such pH gradients.

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