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INTEGRATION OF ALKALINE PHOSPHATASE IN THE INTESTINAL BRUSH BORDER MEMBRANE

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Summary

Alkaline phosphatase has been solubilized from porcine intestinal mucosa by two different methods: treatment of the mucosa by Emulphogen BC 720 and papain hydrolysis of enterocyte brush border membrane vesicles. Two different enzyme forms have been obtained by these methods.

The two enzyme forms ('detergent form' and 'papain form') have been purified to homogeneity by similar techniques and exhibit closely related molecular characteristics. However, the detergent form displays a hydrophobic behaviour and aggregates in media free of detergent. The two forms can be differentiated by their electrophoretic mobility on polyacrylamide gel in the absence of sodium dodecyl sulphate.

By electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate, it has been shown that the detergent and papain forms of alkaline phophatase are dimers consisting of two apparently identical subunits whose molecular weights are 64 000 and 61 000, respectively. The difference between these molecular weights has been attributed to the existence of a hydrophobic region in the detergent form which is present on each subunit.

Introduction

The plasma membrane of the enterocyte brush border is particularly rich in hydrolases. These hydrolases are glycoproteins and can be easily identified by their enzymatic reactions. They consist of aminopeptidases, disaccharides, γ-glutamyltransferase and alkaline phosphatase. They are responsible for the last

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Abbreviations: SDS, sodium dodecyl sulphate; d-alkaline phosphatase, detergent form of alkaline phosphatase; p-alkaline phosphatase, papain form of alkaline phosphatase.

steps of the intraluminar digestion and may be involved in the transport of breakdown products through the membrane [1].

These hydrolases provide excellent models for the study of membrane glycoprotein integration [2,3]. Preliminary observations revealed that these hydrolases could be totally released from the membrane by detergent extraction or by papain hydrolysis. This resulted in two different molecular species which could be easily separated by polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate (SDS) [4].

The enzymes obtained after papain hydrolysis ('papain form') are soluble in aqueous media; those obtained after detergent extraction are hydrophobic and aggregate in aqueous media in the absence of detergent.

The detergent form, which is the intact form of the molecule, is cleaved by papain hydrolysis into two moieties: a hydrophilic part, indistinguishable from the papain form obtained by direct cleavage from the membrane and a small hydrophobic peptide. The papain and detergent forms and the hydrophobic peptide have been purified in the case of pig intestinal and renal aminopeptidases [5—7] and intestinal maltase [6].

However, the exact stoichiometry between the 2 parts of the molecules has not been established. In the case of pig aminopeptidases which are composed of 3 different subunits [5,7], the identification of only one hydrophobic peptide and of only one additional N-terminal residue in the papain form could suggest that only one amongst the 3 subunits bears a hydrophobic part, constituting its N-terminal sequence [6]. But it has been suggested that the existence of 3 different subunits may result from an artefact due to a proteolytic degradation of the native enzyme [8,9]. If this is the case, each of these latter subunits could bear a hydrophobic part. In the case of the γ-glutamyl transferase which is an asymmetrical polymer, it has been shown that only the high molecular weight subunit bears a hydrophobic area [10].

In any event, these hydrolases like other membrane proteins [11—13], are amphipathic molecules composed of two distinct regions. Their hydrophilic moiety, representing the major part of the enzyme, is located at the exterior of the surface of the membrane [14]. It contains the catalytic site and constitutes the papain form of the enzyme. Their hydrophobic region accounting for about 2—6% of the enzyme and situated in the membrane bilayer gives to the molecule striking hydrophobic properties, in particular a strong affinity for lipids [15].

We have attempted to extend this model of an amphipathic protein to other hydrolases. The choice of alkaline phosphatase is justified by the two following considerations. Preliminary studies [4] have shown that alkaline phosphatase, like other hydrolases, can be released as two forms migrating separately during polyacrylamide gel electrophoresis, when membranes are treated with a detergent or with papain. Since by both techniques, the solubilization of the alkaline phosphatase is much more difficult than that of other hydrolases, the existence of a different mode of integration cannot be dismissed. On the other hand, most hydrolases are polymers [5,7,10,16,17], often asymmetrical, but the relation between their oligomeric structure and their activity is unknown. By contrast, alkaline phosphatases from various sources (calf intestine brush border [18], bovine and porcine renal brush border [9,19], human placenta

[20], cow's milk [21] and human liver [22]) are symmetrical dimers, and the enzymatic mechanism strictly adheres to this structural characteristic [23]. Therefore, the study of the integration of the alkaline phosphatase in the membrane seemed to us interesting because it provides a good example of a membrane protein widely distributed throughout the tissues, for which the importance of its oligomeric state to its function is known.

In the present work, purification of alkaline phosphatase into its two forms is described. The fact that the detergent form consists of two identical subunits of higher molecular weight than that of the two subunits of the papain form, has allowed us to show that alkaline phosphatase is integrated in the membrane by two hydrophobic peptides derived from its two subunits.

Materials and Methods

Materials

Ultrogel AcA 34 was from LKB, Sepharose 4B from Pharmacia, DEAE-cellulose (DE-32) from Whatman, Cyanogen bromide, tyramine and 4(*p*-aminophenylazo)phenylarsonic acid from Fluka, Naphtol AS MX phosphate and Fast Red TR salt from Sigma, papain from Boehringer, Emulphogen BC 720 from GAF (France) and Aquacid III from Calbiochem.

Methods

Alkaline phosphatase activity was measured at 37°C with the substrate *p*-nitrophenyl phosphate 5 mM in a 0.1 M Tris buffer (pH 8.5) containing 0.4 M NaCl. Hydrolysis was followed spectrophotometrically at 410 nm. The molar extinction coefficient used was 17 000 $M^{-1} \cdot cm^{-1}$. One unit of activity is defined as that amount of enzyme which hydrolyzes 1 μmol of substrate per min.

7.5% disc gel electrophoresis and the methods used for protein, glycoprotein and alkaline phosphatase specific staining have already been described in detail [4].

5.6% polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Fairbanks et al. [24] after reduction and carboxymethylation of the protein samples [26]. The following proteins were used as standards: bovine serum albumin, 69 000; pancreatic lipase, 48 000; ovalbumin, 43 000; porcine pepsin, 34 000 and bovine trypsinogen, 24 000.

Affinity chromatography was performed by the method of Brenna et al. [27]. The capacity of the Sepharose-tyraminyl-4-azo-phenyl-4-azo-phenyl-arsonic acid (derivative C) for pig intestinal alkaline phosphatase was about 4—5 mg of enzyme retained per 500 ml of gel.

N-terminal residues were identified by a modified version of the dansylation technique [28] and the technique of Edman [29] was used to eliminate the N-terminal residue from reduced and carboxymethylated samples.

Results

(1) Extraction of the detergent form of alkaline phosphatase

Pig intestine (jejunum and ileum) was obtained from the slaughter house

where it was washed with cold phosphate-buffered saline, (10 mM phosphate buffer/0.15 M NaCl, (pH 7.4)). In the laboratory, mucosa was prepared by squeezing the intestine between two rubber rollers and was stored frozen. All subsequent operations were carried out at 4°C.

A preliminary interesting observation was that d-alkaline phosphatase extraction was pH dependent. Indeed, treatment of the mucosa with a buffered Emulphogen solution at pH 6 resulted in the quantitative extraction of aminopeptidase and maltase activities [6], while alkaline phosphatase was poorly extracted. The optimum pH for alkaline phosphatase extractionwas approximately 8—8.5. Therefore, the following procedure was used: frozen mucosa was cut into small fragments, suspended in 2 vols. 10 mM potassium phosphate buffer (pH 6) containing 2% Emulphogen (v/v) and incubated under magnetic stirring at 4°C for 2 h. The supernatant obtained after centrifugation at 14 000 rev./min for 1 h (Beckman, JA 14 rotor) contained only 15—25% of the total alkaline phosphatase activity and was discarded. The pellet was resuspended into 6 vols. water containing 2.5% Emulphogen (v/v) and the pH adjusted to 8.5 with a 0.2 M Tris · HCl buffer, (pH 9). A large amount of detergent was necessary to ensure complete solubilization of alkaline phosphatase and an increase of the volume of the extracting medium was found to be more efficient than an increase of the Emulphogen concentration. The mixture was again gently stirred at 4°C for at least 3 h, then centrifuged at 14 000 rev./min for 1 h in the same rotor as above. The supernatant thus obtained contained nearly all the remaining alkaline phosphatase activity, and was brought up to 45% saturation with ammonium sulfate in order to precipitate the alkaline phosphatase.

The precipitate was solubilized in 10 mM Tris · HCl buffer containing 1% Emulphogen (v/v), 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ and dialyzed for 18 h against 10 l of the same buffer. As reported by several authors [30,31] Zn^{2+} is essential for alkaline phosphatase activity and Mg^{2+} stimulates the activity. These two cations exert a stabilizing effect on the enzyme during the purification steps [22]. Indeed, in their absence, important and irreversible losses of activity were consistently observed during dialysis. Therefore, after the ammonium sulfate precipitation step, these two cations were included in all solutions. The yield at this step was reproducibly 50—60%.

(2) Solubilization of p-alkaline phosphatase by papain

In contrast with experiments with aminopeptidase, the detergent form was highly resistant to proteolytic digestion and in all attempts to obtain the soluble form of the enzyme from the partially purified detergent form, the yield was less than 10%. It may be assumed that extraction from the membrane causes some conformational change which renders the protein resistant to digestion by papain. Therefore, the soluble form of alkaline phosphatase was obtained, routinely, by papain hydrolysis of brush border membrane vesicles.

The vesicles were prepared as previously described [24], except that the last gradient purification step was omitted. The membrane suspension contained about 30—40% of the alkaline phosphatase activity of the starting mucosa and was stored frozen until use, since it was shown that papain hydrolyzes freeze-thawed membranes better than fresh preparations. After thawing, the

membrane suspension was washed before hydrolysis with 10 mM Tris · HCl buffer (pH 7.3), containing 0.15 M NaCl, in order to eliminate the Mg^{2+} present in the media for the preparation of the membranes, as it is known that divalent cations inhibit papain activity.

Papain hydrolysis of the membranes was performed as follows: 3 ml of papain solution (10 mg/ml) diluted in 30 ml 0.1 M citrate buffer (pH 6.2), were activated for 15 min at 0°C in the presence of 0.3 mM dithiothreitol and 5 mM cysteine. Pre-activated papain was mixed with 30 ml of washed membranes (30 mg protein/ml) suspended in citrate buffer, the pH was adjusted to 6.2 if necessary and the mixture was incubated for 1 h at 37°C under frequent shaking. Hydrolysis was stopped by dilution with 3 vols. of 50 mM Tris · HCl buffer (pH 8.5) and the membranes were sedimented by centrifugation at 105 000 × *g* for 1 h (Spinco L 250, Rotor 42 Ti). The supernatant contained the p-alkaline phosphatase with a yield of approximately 70—85% and no inhibition of activity at this step. This supernatant was dialyzed for 18 h with one change against 10 l of 10 mM Tris · HCl buffer (pH 8.4), containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$.

(3) Purification of the two forms of alkaline phosphatase

The two forms of alkaline phosphatase display a very similar behaviour during the different steps of the purification and, therefore, only the purification scheme for the p-alkaline phosphatase will be described in detail with the modifications necessary for d-alkaline phosphatase mentioned when needed.

p-Alkaline phosphatase purification. Affinity chromatography: The supernatant obtained after papain hydrolysis and dialysis was loaded onto the Sepharose derivative column. The elution profile is given in Fig. 1A. Active fractions were pooled and dialyzed against 10 l of 10 mM Tris · HCl buffer (pH 8.4) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, for 18 h. The volume of the pooled fractions was about 350 ml. The specific activity was increased 50-fold by this affinity-chromatography step although there were still many contaminating proteins present as judged by polyacrylamide gel electrophoresis.

DEAE-cellulose chromatography: The active material was then chromatographed on a DEAE-cellulose as described in Fig. 1B. The elution with phosphate appeared to be specific and this ion exchange step provided for a 2—4-fold increase in the specific activity. The eluate from the DEAE-cellulose column was concentrated to 3—5 ml at room temperature with aquacide III (flake polyethylene glycol).

Ultrogel filtration: The final step was a gel filtration on a Ultrogel AcA 34 column (Fig. 1C). Alkaline phosphatase emerged as a symmetrical peak corresponding to about 1.4 void volume. The fractions with the highest specific activity were pooled and concentreated at room temperature with aquacide to approximately 1 mg protein/ml. The specific activity of the purified protein was 230 units per mg protein *.

d-Alkaline phosphatase purification. All the media contained 1% Emulphogen

* Assay conditions vary considerably in the literature, and as alkaline phosphatase activity varies considerably with pH, temperature and choice of buffer [32], it is difficult to compare the specific activity obtained here with that published by other authors.

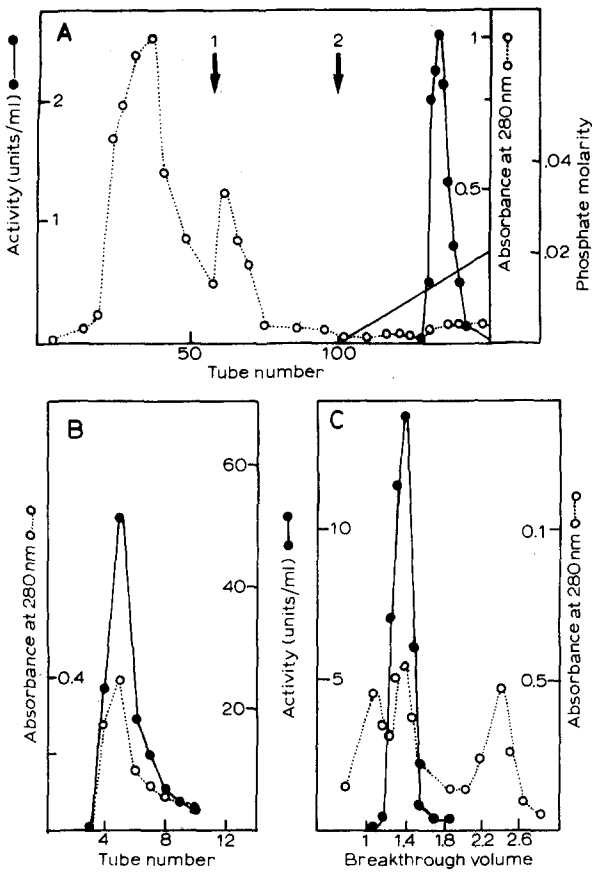

Fig. 1. Main steps of p-alkaline phosphatase purification. A. Affinity chromatography on Sepharose 4B coupled with 4(*p*-aminophenylazo)phenylarsonic acid. 500 ml of the Sepharose derivative were poured into a 6 X 25 cm column, equilibrated with a 10 mM Tris · HCl buffer (pH 8.4) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ and loaded with 300 ml of dialyzed papain supernatant (total activity, 750 units; total proteins, 1 g). The column was washed with 1.5 l of the same buffer, then the concentration of the buffer was raised to 100 mM (arrow 1). Specific elution of alkaline phosphatase was performed with a linear phosphate gradient (0—40 mM) in the 100 mM Tris · HCl buffer (2 X 800 ml) (arrow 2). Fraction volume, 28 ml. Fractions 126 to 134 were pooled. B. DEAE-cellulose chromatography. The pooled fractions from the Sepharose column were adsorbed after dialysis on a small DEAE-cellulose column (1 X 6 cm) equilibrated with a 10 mM Tris · HCl buffer (pH 8.4), containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. After washing the column with the same buffer, alkaline phosphatase activity was eluted with 0.1 M potassium phosphate in the buffer. Fraction volume, 2.8 ml; flow rate, 50 ml/h. C. Ultrogel filtration. The pooled fractions from the DEAE-cellulose step (500 units, about 4 mg protein in 5 ml) were concentrated, layered on a Ultrogel AcA 34 column (1.5 X 200 cm) equilibrated with a 10 mM Tris · HCl buffer (pH 8.4) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, and eluted with the same buffer. Fraction volume, 1 ml; flow rate, 5 ml/h.

(v/v). In the affinity step, the Sepharose derivative column was loaded with the dialyzed supernatant obtained by dissolving the ammonium sulfate precipitate (see above) in the buffer. To obtain a good fixation of the d-alkaline phosphatase, it was necessary to dilute the supernatant with at least 1 l of buffer containing 1% Emulphogen (v/v). This large amount of Emulphogen probably impairs the formation of mixed micelles of alkaline phosphatase with other proteins.

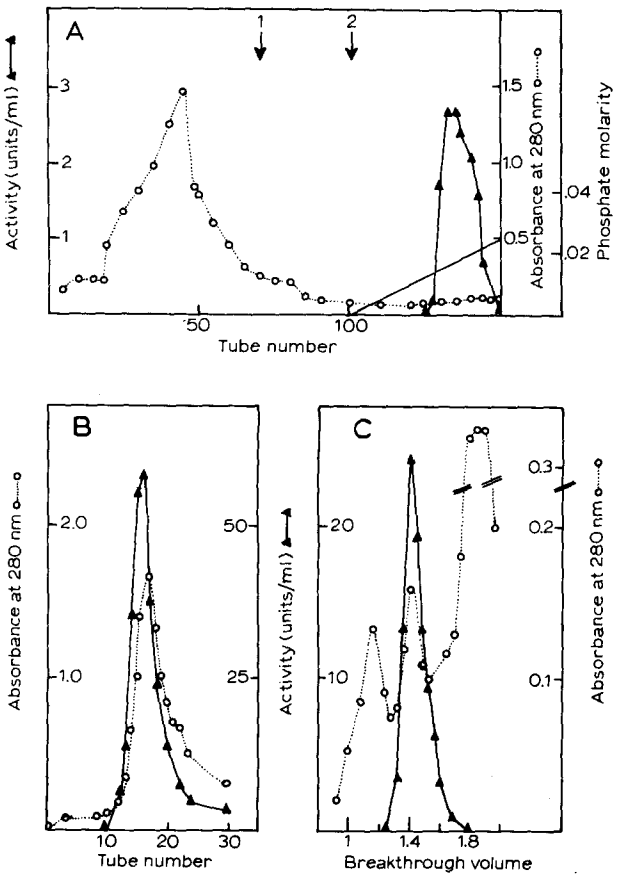

Fig. 2. Main steps of d-alkaline phosphatase purification. A, affinity chromatography; B, DEAE-cellulose chromatography; C, Ultrogel filtration. All conditions are the same as in Fig. 1 except that all buffers contain 1% Emulphogen (v/v).

During the specific elution with the phosphate gradient, d-alkaline phosphatase was eluted at a phosphate concentration between 0.014 and 0.020 M (Fig. 2A) very close to that at which p-alkaline phosphatase was eluted (0.012—0.016 M).

Recovery of the d-alkaline phosphatase was very poor after the DEAE-cellulose chromatography step (Fig. 2B). The yield could be increased by increasing the Emulphogen concentration in the buffer but this caused the elution of a gel-like contaminant which was then very difficult to eliminate.

The elution volumes of the detergent form (Fig. 2C) and the papain form (Fig. 1C) from the Ultrogel column were identical, indicating very similar molecular weights. The specific activity of d-alkaline phosphatase was only 170 units per mg protein, although no contaminating proteins were revealed after electrophoresis on polyacrylamide gel.

Table I summarizes the main steps of the purification of the two forms of pig intestinal alkaline phosphatase. In spite of the use of an affinity step, the yield was low (10—15%. 1.5 mg of pure enzyme was obtained from 250 g of

TABLE I

FLOW-SHEET OF THE PURIFICATION OF THE TWO FORMS OF ALKALINE PHOSPHATASE

The preparation of the detergent form and papain form of alkaline phosphatase was made from 250 g intestinal mucosa.

Fractions	Detergent form			Papain form		
	Total activity (units)	Yield (%)	Specific activity (units/mg protein)	Total activity (units)	Yield (%)	Specific activity (units/mg protein)
Water Homogenate	2672	100	0.03	—	—	—
Pellet after pH 6 Emulphogen extraction	2000	75	—	—	—	—
Supernatant after pH 8.5 Emulphogen extraction	1862	70	0.07	—	—	—
Ammonium sulfate precipitation (0.45 saturation)	1725	65	0.09	—	—	—
Dialysis	1474	55	0.14	—	—	—
Sucrose Homogenate	—	—	—	2544	100	0.03
Membranes	—	—	—	786	31	0.59
Papain supernatant	—	—	—	670	26	—
Dialysis	—	—	—	742	29	0.87
Sepharose affinity column	1363	51	10.0	634	25	44.0
DEAE-cellulose	667	25	40.0	400	16	130.0
Ultrogel filtration	471	17	170.0	300	11	230.00

intestinal mucosa. The purified enzyme could be stored at 0°C for several weeks without loss of activity.

(4) Characteristics of the two forms of alkaline phosphatase

Analysis of purity. The two forms of alkaline phosphatase corresponded to proteins having different behaviours and they could easily be separated by polyacrylamide gel electrophoresis in the absence of SDS. The gel was prepared in the presence of Emulphogen (0.15%, v/v) to impair the aggregation of the d-alkaline phosphatase. The presence of detergent in the gel and in the loading buffer did not change the migration of the p-alkaline phosphatase. Each form of the enzyme gave only one protein band after staining the gels with Coomassie Blue. After electrophoresis in 7.5% gels, the R_F values were 0.39 for the papain form and 0.17 for the detergent form (Fig. 3). The protein bands revealed by Coomassie Blue had exactly the same R_F values as the pink bands found after specific staining for alkaline phosphatase activity (reaction with β-naphtol and Fast Red). Moreover, specific staining with the Schiff reagent has shown that pig intestine alkaline phosphatase is a glycoprotein, as are other alkaline phosphatases. The partially purified detergent form could be slightly hydrolyzed by papain, as noticed above. This could be visualized on polyacrylamide gels by the appearance of a band with an R_F of 0.39. This band corresponded exactly to the band of the papain form and stained specifically for alkaline phosphatase.

Molecular weight and subunit structure. The molecular weight of p-alkaline phosphatase was determined by analytical centrifugation in a Spinco-Beckman

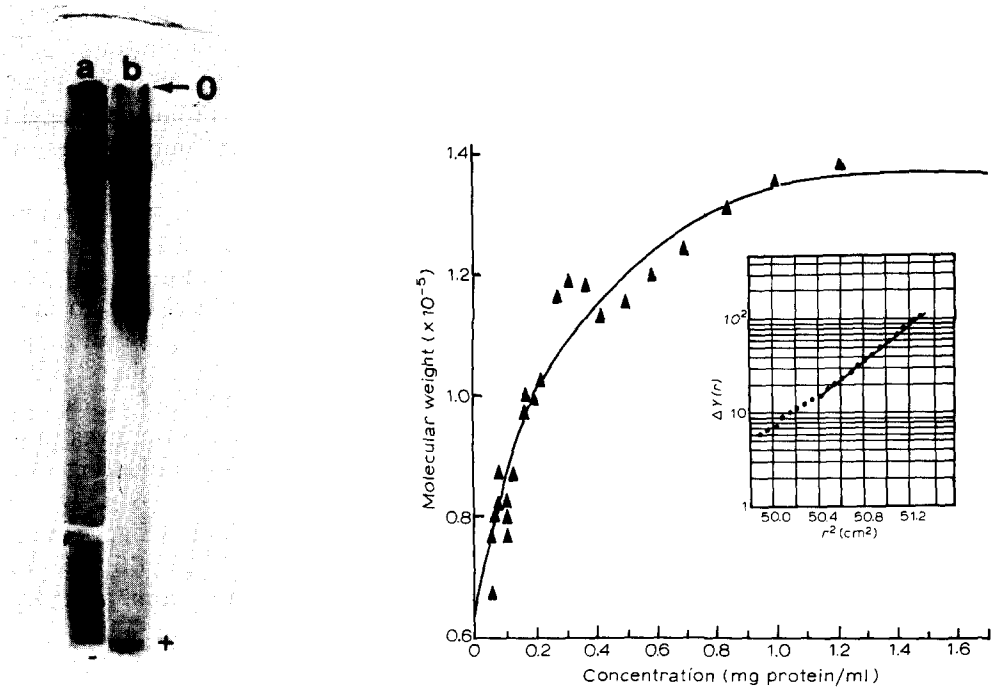

Fig. 3. 7.5%polyacrylamide gel electrophoresis in the presence of 0.15%(v/v) Emulphogen, of d-alkaline phosphatase (a) and a mixture of d- and p-alkaline phosphatases (b).

Fig. 4. Molecular weight distribution as a function of concentration observed for p-alkaline phosphatase in a 50 mM Tris · HCl buffer (pH 8.4) during high speed equilibrium ultracentrifugation assays according to Yphantis [34—36]. The concentration in mg/ml is deduced from fringe concentration. Initial concentration of the sample 0.4 mg/ml. Insert, classical log $\Delta y/r^2$ plot derived from ultracentrifugation.

analytical centrifuge Model E equipped with a Schlieren optical system. The absorption coefficient was determined by interference optics using the same centrifuge with a solution of alkaline phosphatase containing 0.4 mg protein/ml and dialyzed just before use against a 50 mM Tris · HCl buffer (pH 8.4). The mean value for the extinction coefficient at 280 nm ($E^{1\%}_{1cm}$) was 9.56. This value is somewhat higher than those found for the alkaline phosphatases from pig kidney: 7.4 [33] and calf intestine: 7.6 [18].

After recovery, the same sample of protein solution was used for the determination of its molecular weight, by the equilibrium method of Yphantis [34]. The solution was spun at 18 000 rev./min for 26 h at 20°C. The partial specific volume was directly measured with the aid of a microdensitometer (Model 120 C, A, Parr, Austria) and was found to be 0.73 ml/g.

As shown in the insert of Fig. 4, the curve log $\Delta y/r^2$ did not fit exactly with a straight line. Indeed, the molecular weight was observed to vary with the concentration of the protein solution (Fig. 4) and the existence of an equilibrium between a dimeric form (at high concentration) and a monomeric form (at low concentration) was demonstrated. Such an equilibrium between monomeric-dimeric forms or dimeric-tetrameric forms depending on the concentration [35,36] or the pH [20] has already been described for other proteins. The

molecular weight of the dimer and the monomer were, respectively, 135 000 and 65 000, and a molecular weight of approximately 120 000 daltons could be estimated from the straight line derived from the log $\Delta y/r^2$ plot (insert Fig. 4). For the d-alkaline phosphatase an estimation of the molecular weight was made by gel filtration on a calibrated Ultrogel column, and a value of 125 000 daltons was found.

The number of subunits and the molecular weight of each form of alkaline phosphatase has been determined by using polyacrylamide gel electrophoresis in the presence of SDS, after reduction and carboxymethylation of the proteins. As observed by others [9,37], alkaline phosphatase is resistant to SDS and complete denaturation is achieved only in the presence of urea and high concentration of β-mercaptoethanol. Fig. 5B shows a photograph of SDS-polyacrylamide gels of the papain form (a), the detergent form (b) and a mixture of the two forms (c). Each form of alkaline phosphatase gives only one band, thus confirming the purity of the preparations and indicating that the enzyme is made of only one type of subunit.

The molecular weights obtained after calibration of the gels (Fig. 5) were 60 000—61 000 for the subunit of the papain form and 63 000—65 000 for that of the detergent form. These values are extreme values from 5 experi-

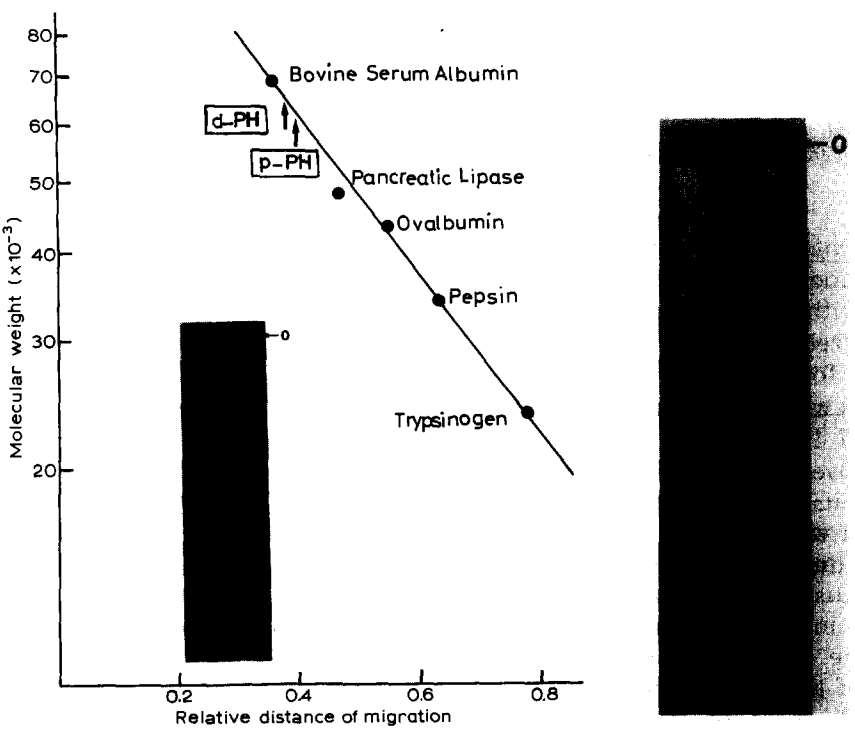

Fig. 5. Molecular weights of the subunits of the two forms of alkaline phosphatase as determined by polyacrylamide gel electrophoresis in the presence of SDS according to Fairbanks et al. [25]. Logarithm of molecular weights are plotted versus relative length of migration. Photograph of the gels: (a) papain form, (b) detergent form, (c) mixture of equal amounts of papain and detergent form.

ments. By SDS-gel electrophoresis of a mixture of the detergent and papain forms of alkaline phosphatase two distinct bands clearly separated (see gel c of Fig. 5B). As non-denatured alkaline phosphatase had a molecular weight close to 120 000—130 000, it may be concluded that the enzyme is made of two, apparently identical subunits.

Determination of N-terminal residues. The nature of the N-terminal amino acid residues was determined on the two forms of alkaline phosphatase. After dansylation, each form gave only one dansyl-amino acid: phenylalanine. After elimination of this N-terminal residue by the Edman technique, the second amino acid was found to be isoleucine. Thus, the N-terminal sequence was identical in both forms of alkaline phosphatase and in both subunit of each form.

Discussion

Three intestinal alkaline phosphatases have already been purified to homogeneity from calf [18], rat [38] and human intestine [39]. Indeed, the intestine, together with the placenta, is one of the richest sources of alkaline phosphatase activity [40]. However, the purification was hampered by the very small amount of enzyme present in the tissues, and furthermore, this enzyme appears to be tightly bound to the membrane. In the methods so far published, this association was destroyed either by tissue autolysis or, more frequently, by delipidation with *n*-butanol [40]. In most cases, only the hydrophilic form or 'papain form' in our nomenclature was obtained since the extraction of treated tissues and the purification were made in the absence of detergent. Some authors have nevertheless, obtained aggregating forms in addition to the soluble form [41]. This would indicate a conservation of the hydrophobic domain in some molecules.

While this manuscript was in preparation, the calf thymus enzyme was purified after Triton extraction [42]. However, the authors were only interested in the enzymatic properties of the alkaline phosphatase obtained.

The aim of this work has been the purification of the alkaline phosphatase after papain solubilization and detergent extraction and the comparison of the structure of the 2 forms of the enzyme thus obtained. As in the case of the other brush border hydrolases [2—3] and other membranous glycoproteins [11—13], the difference between the enzymes extracted by the two methods resides in the hydrophobic part of the molecule than anchors the protein to the membrane.

As for other hydrolases, the two forms of alkaline phosphatase, characterized by their electrophoretic mobility could be purified by the same procedure, provided that the medium contained 1% Emulphogen (v/v) in the case of the detergent form of the enzyme. The two forms were eluted by approximately the same ionic strength from DEAE-cellulose column and emerged after about the same elution volume from Ultrogel column. Therefore, one might expect the two molecular forms to have a similar charge and molecular weight. The molecular weight, as determined by gel filtration for d-alkaline phosphatase and by the method of Yphantis for the p-alkaline phosphatase, was in the range of 120 000—130 000. The separation of the two forms of

alkaline phosphatase was not possible by gel filtration. By polyacrylamide gel electrophoresis in the presence of SDS, it has been shown that the two forms of alkaline phosphatase are dimers, composed of identical subunits. This result is not surprising since this dimeric symmetry is an essential feature for the activity of alkaline phosphatases [23].

An interesting point is that the subunits of the two forms of alkaline phosphatase have, in fact, slightly different molecular weights. The difference, although small, is significant, and indeed, when a mixture of the two forms is submitted to polyacrylamide gel electrophoresis in the presence of SDS, two clearly separated bands are obtained. A likely hypothesis is that this difference of 3000—4000 in the molecular weight is due to the presence of a hydrophobic area in the detergent form which is absent in the papain form. The molecular weight of the hydrophobic part may be underestimated because of the lack of resolution of the polyacrylamide gel in the region of 60 000 daltons. Whatever the exact molecular weight it can be concluded that, as previously found for other intestinal hydrolases, the hydrophobic domain of alkaline phosphatase appears to be very small compared to the hydrophilic moiety. However, these hydrophobic sequences which represent 2—3% of the total molecular weight of the hydrolases could be responsible for the special behaviour of the detergent forms.

By polyacrylamide gel electrophoresis in the presence of SDS, only one kind of subunit has been found for the detergent form which implies the presence of a hydrophobic peptide on each subunit. This result is interesting since it shows for the first time how a dimeric enzyme is integrated into the membrane. This anchorage of alkaline phosphatase in the membrane by two points must assign some rigidity to the enzyme and a greater stability to the integrated system. This fact, together with the existence of specific binding of certain phospholipids [43] could explain the difficulty encountered in detaching alkaline phosphatase from the membrane.

The identity of the N-terminal dipeptide (Phe-Ile) in the subunits of the papain and detergent forms suggests that the hydrophobic peptides are not located on the N-terminal side, as it is apparently the case for the aminopeptidases from porcine [6] and rabbit (Ferracci, H., personal communication) origin and the maltase from porcine mucosa [6]. If alkaline phosphatase is anchored in the membrane by its C-terminal end, this could imply that the modes of integration in the membrane, of proteins belonging to the same class (e.g. hydrolases), are not identical.

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