DIFFERENTIAL LOCALISATION OF PHOSPHORYLATED AND NON-PHOSPHORYLATED FORMS OF ARYLSULFATASE A IN LYSOSOMES

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1. Introduction

The phosphohexosyl components of the lysosomal acid hydrolases are believed to form a part of the common recognition marker for the receptor mediated uptake of these glycoproteins by fibroblasts [1–5]. Depending upon the rate of uptake of a particular enzyme by cultured cells, a 'high uptake' form may be distinguished from a 'low uptake' form of the same enzyme in the same system [6]. The 'high uptake' form of β-glucuronidase [7], which is efficiently and specifically pinocytosed by fibroblasts, is more acidic than the 'low uptake' or poorly pinocytosed form; alkaline phosphatase treatment of the former enzyme abolishes its 'high uptake' behaviour without diminishing its catalytic activity.

Most lysosomal hydrolases are known to exist in two states, the membrane-bound state and the free (non-latent) state [8]. The physiological significance of these two states is poorly understood. Here we show that the phosphorylated high uptake form of arylsulfatase A (arylsulfate sulfohydrolase EC 3.1.6.1) from sheep brain is a membrane-bound enzyme whereas the free non-phosphorylated 'low uptake' form is a soluble protein. This is perhaps the first report demonstrating that the high and low uptake forms of a lysosomal enzyme may be correlated with the physiological microenvironments in which these enzymes exist.

2. Materials and methods

DEAE-cellulose (DE-52, microgranular) was the product of Whatman Co. Acrylamide and *N,N'*-bis acrylamide were obtained from Eastman Organic Chem. *P*-Nitrocatecholsulfate, *Cl. perfringens* neuraminidase and calf intestine alkaline phosphatase were obtained from Sigma Chemical Co. One unit of enzyme is defined as the amount of enzyme required to form 1 μmol product/min.

2.1. *Separation and solubilization of membrane-bound arylsulfatases*

The lysosomal fraction from adult sheep brain was prepared as in [9]. Crude mitochondrial fraction which also contained lysosomes was suspended in 10 mM Tris–HCl (pH 7.4). It was frozen and thawed 6–8 times and was then centrifuged at 100 000 × *g* for 60 min at 4°C to separate the soluble fraction from the membrane fraction. Enzyme activity associated with this supernatant was termed the free form of the enzyme and that associated with the sediment was termed the bound form. The contaminant free enzyme associated with the pellet was removed by washing 3 times with 10 mM Tris–HCl (pH 7.4). The butanol extraction procedure for the solubilization of the membrane-bound enzymes was carried out with washed pellet at pH 6.0 by the method of [10] as described in [11].

2.2. *Assay of enzymes*

Arylsulfatases A and B were assayed as in [12] as modified [13]. Total arylsulfatases were assayed with 10 mM *p*-nitrocatecholsulfate in 0.5 M acetate buffer (pH 5.0). Protein was determined as in [14] using crystalline bovine serum albumin as standard. One unit of enzyme is defined as that amount of enzyme required to produce 1 nmol nitrocatechol/min under the standard assay conditions. The specific activity is expressed as no. units/mg protein.

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2.3. *Ion-exchange chromatography*

The free and solubilized enzyme preparations were dialyzed overnight at 4°C against 1000 vol. 10 mM Tris–HCl (pH 7.4), subsequently cleared by centrifugation whenever necessary, and were then applied to a column of DEAE-cellulose (5 × 0.52 cm) equilibrated with 10 mM Tris–HCl (pH 7.4). The column was washed with 10 ml column buffer containing 50 mM NaCl. A linear gradient of 20 ml total vol. from 50–250 mM NaCl in equilibrium buffer was then applied to the column, and 0.5 ml fractions were collected. The enzyme activities were assayed as above.

2.4. *Treatment with neuraminidase and alkaline phosphatase*

The concentrated and dialyzed enzyme preparations from DEAE–cellulose eluates were incubated with neuraminidase (the enzyme protein:neuraminidase ratio being 1 mg:2 × 10^{-3} units) in 20 mM Tris–acetate (pH 6.0) for 1 h at 37°C and with alkaline phosphatase (the enzyme protein:alkaline phosphatase ratio being 1 mg:5 × 10^{-3} units) in 10 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$ for 30 min at 37°C. In the case of neuraminidase treatment, the precipitable protein was removed by centrifugation and the clear supernatant was dialyzed against 10 mM Tris–HCl (pH 7.4) at 4°C. These dialyzed preparations were used for electrophoretic studies.

2.5. *Periodate oxidation*

Periodate oxidation was done as in [15].

2.6. *Gel electrophoresis*

The gel electrophoretic patterns of bound and free arylsulfatase A were studied on 7.5% polyacrylamide gels [16] with 4 mM histidine buffer (pH 7.0) as gel buffer and 41 mM sodium citrate buffer (pH 7.0) as bridge buffer. A current of 3 mA/tube was applied for 3 h. Red bands with arylsulfatase A activity were visualized by incubating the gels in 10 mM *p*-nitrocatecholsulfate in 0.5 M acetate buffer (pH 5.0), for 45 min at 37°C and then transferring into 1 N NaOH for 2–3 min.

3. Results

3.1. *Solubilization of membrane-bound enzyme*

By repeated freezing and thawing, ~80% of the total arylsulfatases (arylsulfatases A and B) were released into the supernatant whereas the remaining 20% was associated with the membrane fraction. Solubilization of the membrane-bound arylsulfatases was affected by butanol and maximum solubilization was achieved with 30% butanol. There is a 5-fold increase in arylsulfatases activity after solubilization. The free and the solubilized enzymes were purified 150- and 500-fold, respectively, over the homogenate.

3.2. *Ion-exchange chromatography*

When the enzyme preparations from both free and solubilized fractions were loaded into DEAE-cellulose column, they were resolved into two enzyme peaks (fig.1). In both the cases, ~58% of the total enzyme recovered from the column came out in low salt wash whereas the remaining 42% was eluted by high salt. In the case of free enzyme, the second peak of enzyme activity was eluted earlier compared to that of solubilized enzyme. When the fractions constituting the second peak of enzyme activity from both free and solubilized enzyme preparations were pooled together, mixed and applied to DEAE-cellulose column, two peaks corresponding to the free and solubilized enzyme preparations were again obtained (data not presented). This suggests that the difference in the elution profiles of the second peak in both the cases were not due to artifact.

The peak I and peak II enzymes were characterized as arylsulfatases B and A, respectively, by studying the inhibition pattern with pyrophosphate and NaCl. This was further confirmed by the use of silver nitrate

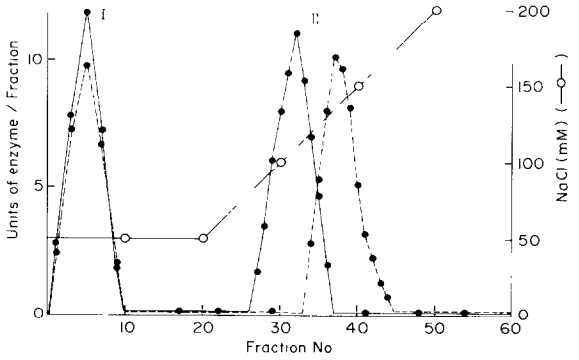

Fig.1. The elution profile of arylsulfatases through DEAE-cellulose column. (–) 4.5 mg (190 units) free and (– – –) 1.4 mg (186 units) solubilized enzyme protein were loaded into the column. The enzyme activity was assayed as in the text.

Table 1
Polyacrylamide gel electrophoretic patterns of arylsulfatase A after different treatments

Type of enzyme	Treatment	Electrophoretic mobility (Distance traversed from cathode to anode in cm)
Solubilized	None	4.9
Solubilized	Alkaline phosphatase	2.3
Solubilized	Alkaline phosphatase + Na_2HPO_4 (1 mM)	4.9
Solubilized	Periodate oxidation	2.2
Free	None	2.3
Free	Alkaline phosphatase	2.3

Conditions for gel electrophoresis, staining of arylsulfatase A and also the alkaline phosphatase treatment of the enzyme are in the text. For periodate oxidation 2.28 units enzyme in 50 mM Tris–acetate (pH 6.0) were subjected to oxidation with 30 mM Na-metaperiodate at 4°C in the dark for 4 h. The reaction was terminated by addition of excess ethylene glycol and soluble oxidation products as well as residual reagents were removed by dialysis against the same buffer Total length of all the gels were 7 cm and distance was measured from the top of the gel to the centre of enzyme stained bands. Alkaline phosphatase treatment was carried out as in the text

[17]. Peak II enzyme was inhibited to 80% by 1 mM $AgNO_3$ whereas under identical conditions, peak I enzyme activity were not affected. By using DEAE-cellulose chromatography, arylsulfatase A from free and solubilized preparations was purified 3-fold over the previous step and total purification achieved was 450- and 1500-fold over the homogenate.

3.3. *Electrophoretic studies*

These highly purified enzyme fractions obtained from DEAE-cellulose chromatography were used for all electrophoretic studies. Table 1 gives the electrophoretic mobilities in polyacrylamide disc electrophoresis of both free and solubilized forms of arylsulfatase A. The solubilized form of the enzyme was found to have more anodic mobility than the free form which conforms with the observation of ion-exchange chromatographic studies. Treatment with alkaline phosphatase significantly reduced the anodic mobility of solubilized arylsulfatase A and it almost corresponded to the position of that of the free form whereas the mobility of the free form remained unchanged, suggesting thereby that the more negative charge of the solubilized enzyme is probably due to phosphoprotein nature of the enzyme and phosphate group is present as monoester linkage. That the decreased electrophoretic mobility of the solubilized enzyme is due to the removal of phosphate groups and not due to the action of any contaminant proteolytic enzyme in alkaline phosphatase has further been confirmed by the observation that alkaline phosphatase treatment in presence of Na_2HPO_4 caused no change in mobility of the bound enzyme (table 1). The periodate oxidation also reduced the mobility of the solubilized enzyme (table 1). The same extent of reduction in mobility of the solubilized enzyme after periodate oxidation and alkaline phosphatase treatment suggests that all the phosphate groups may be exclusively attached to the carbohydrate part of the glycoprotein enzyme. Neuraminidase treatment did not cause a significant decrease in the mobilities of free and solubilized enzymes.

4. Discussion

Although the crude mitochondrial fraction containing lysosomes was used here it is well-known that arylsulfatase A is found exclusively in lysosomes [18] and the inferences drawn may be considered as valid as those obtainable from a pure lysosomal preparation.

Arylsulfatase A is shown here to be present in two forms, a membrane-bound phosphorylated form and a free non-phosphorylated form. The phosphorylated forms of lysosomal hydrolases are the 'high uptake' forms, the sugar-linked phosphates partici-

pating in the specific binding of these enzymes to plasma membrane receptors which aid in their internalization into lysosomes. Our finding that only the membrane bound form of arylsulfatase A is phosphorylated suggests that this specific sugar phosphate–receptor complex remains associated within the lysosomal milieu. The additional observations that:

(i) There is a 5-fold increase in membrane bound enzyme after solubilization;
(ii) Concentration of free non-phosphorylated form of the enzyme is 4 times more than membrane bound enzyme;

suggest that soluble form of the enzyme is physiologically important catalytic form and membrane bound form is a 'transport intermediate'. In the intact lysosomes some dephosphorylation apparatus may exist which converts the latent membrane-bound enzyme to a soluble catalytically active protein.

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