PURIFICATION OF THE HUMAN URINARY GLYCOPROTEIN WITH GASTRIC ANTISECRETORY ACTIVITY BY 'SUBUNIT EXCHANGE CHROMATOGRAPHY'

Giacomo CARREA, Giuseppe LUGARO, Riccardo NIADA*, Paola VECCHINI+ and Eraldo ANTONINI+

*Laboratorio di Chimica degli Ormoni del CNR, Via Mario Bianco 9, I-20131 Milano, *Crinos Biological Research Laboratories, 22079 Villa Guardia* and *+Istituto di Chimica, Facoltà di Medicina dell'Università di Roma, Italy*

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1. Introduction

A glycoprotein with marked gastric antisecretory activity (human urinary gastric inhibitor) has been isolated in our laboratory from human urine [1,2]. This inhibitor is made of identical subunits whose molecular weight was evaluated to be ~15 000 by gel-filtration and disc-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) and by C-terminal analysis [3]. Preliminary experiments have suggested that the molecular weight of the glycoprotein depends on pH due to a reversible association–dissociation process.

It has been shown recently [4–6] that, in the case of an associating–dissociating protein system, the protein subunits immobilized on a solid matrix will interact with the subunits in solution. This interaction can be measured quantitatively and can be used to analyze the association–dissociation properties of the system under various conditions. It also provides a powerful purification tool because of its specificity.

The process which has been called 'subunit exchange chromatography' has been already applied to various proteins [4–6]; the results obtained with the gastric antisecretory glycoprotein are reported here.

2. Materials and methods

2.1. *Ultracentrifugation analyses*

Sedimentation velocity experiments were carried out near 10°C at 44 000, 52 000 and 56 000 rev./min using a Spinco model E ultracentrifuge equipped with a temperature control unit. The sedimentation coefficients were calculated by a least-squares procedure and corrected to 20°C in water ($s_{20,w}$) in the conventional way using tabulated values of viscosity and density; they are expressed in Svedberg units. The buffers were: sodium phosphate buffer, $I = 0.1$ M, in the neutral region, sodium acetate buffer, $I = 0.1$ M in the acid region and carbonate–sodium hydroxide buffer, $I = 0.1$ M at pH 9–11. In order to evaluate the percentage of each component as a function of pH, the areas under the Schlieren peaks were traced on tracing paper with a 10-fold magnification and measured by planimetry.

2.2. *Immobilization of the inhibitor*

The coupling reaction was conducted at 2°C for 6 h. The inhibitor was attached to CNBr-activated Sepharose 4B [7] under different pH conditions, extent of activation and with or without the presence of denaturants. Glycine was added 6 h later for quenching unreacted groups. The samples were then washed exhaustively with 0.1 M phosphate buffer (pH 8) and with 8 M urea to remove any protein not covalently linked to the matrix, then equilibrated in the proper buffer.

The amount of protein immobilized on the matrix was determined by amino acid analysis of known volumes of settled Sepharose after hydrolysis with HCl (final conc. 6 N) for 24 h at 110°C.

2.3. *Binding of inhibitor to inhibitor–Sepharose*

The assay was performed in a recycling thermostated reactor in which the beads of Sepharose-immobilized protein were gently stirred [6]. The slurry was usually made of 2 ml gel and 3 ml buffer. Concentrated

solutions of the free protein were added stepwise and the A_{280} of the solutions leaving the reactor through the porous glass was measured in a constant-flow microcell. From this measure the amount of protein bound by the immobilized inhibitor was calculated.

2.4. *Purification of the inhibitor by 'subunit exchange chromatography'*

Aliquots (1 l) of pooled urines from pregnant women were extracted with benzoic acid according to [8]. The material so obtained (~200 mg) was extracted with 20 ml 0.1 M Na-acetate buffer (pH 5.4) and centrifuged. The supernatant was chromatographed on a column of Sepharose-immobilized inhibitor.

3. Results and discussion

Analytical ultracentrifugation experiments showed that the inhibitor behaves as a reversible associating–dissociating system very sensible to pH variations.

At neutral pH, 2 peaks were present with $s_{20,w}$ = 8 S (~60% of the total) and $s_{20,w}$ = 3 S (~40%), respectively. In the alkaline pH range (pH 10–11) the relative amount of the 3 S peak increased and occasionally was the only peak present. At pH 5.4 a single peak with $s_{20,w}$ = 11 was found. In 6 M urea (pH 7) a single $s_{20,w}$ = 3.7 peak was present.

The association of the inhibitor over a wide range of conditions was studied using the immobilized glycoprotein.

A good correlation was found between the interaction of the inhibitor with the inhibitor–Sepharose and the behaviour of the inhibitor in the ultracentrifuge.

The immobilized inhibitor showed a decreasing capacity to bind free inhibitor on increasing pH (from pH 5.4–10), ionic strength (from 0.05–0.3 M Na-acetate) and temperature (from 5–35°C). For instance the binding capacity was ~5-times lower at pH 10 than at pH 5.4 and ~30% lower at 35°C than at 5°C. The expected correlation of the amount bound with the concentration of free glycoprotein was found (fig.1). The equilibrium was attained within 30–40 min.

The immobilization conditions (table 1) influenced the binding capacity of inhibitor–Sepharose derivatives; the binding capacity was expressed as *Ib/Is* (see

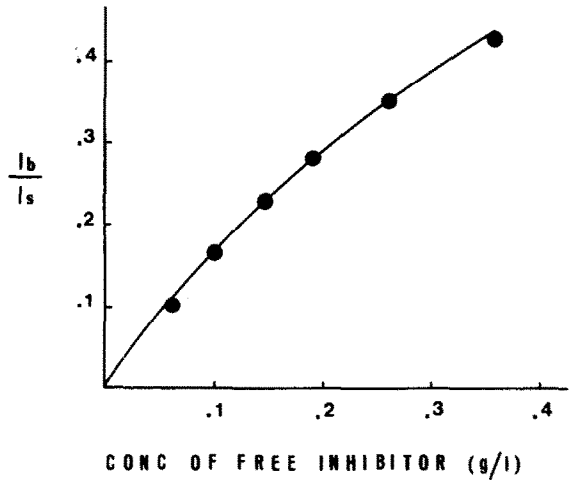

Fig.1. Binding of free inhibitor to inhibitor–Sepharose (immobilization at pH 10.2; see table 1). In ordinate the ratio between the amount of inhibitor bound by inhibitor–Sepharose (*Ib*) and the amount of covalently linked inhibitor (*Is*). Buffer: 0.1 M sodium acetate (pH 5.4); temp. 25°C.

legend to fig.1). A correlation with the pH of immobilization was found. The derivative obtained at pH 10.2 had >2-times the binding capacity of that obtained at pH 6. This should be due to the fact that at high pH the inhibitor is immobilized in dissociated form more suitable to interact with the free glycoprotein. Derivatives obtained in the presence of urea and SDS showed however low binding capacity, possibly because the inhibitor was attached in an irreversible denaturated form. The extent of activation of the matrix had no effect.

The interaction between immobilized inhibitor

Table 1
Effect of coupling conditions on the amount of inhibitor immobilized on Sepharose

pH of immobilization[a]	mg CNBr/ml settled Sepharose	mg attached inhibitor/ml settled Sepharose
6	60	2.5
8.5	60	2.7
10.2	60	2.6
8.5	10	0.7
8.5, 0.5% SDS	60	1.7
8.5, 6 M urea	60	1.8

[a] 4 mg free inhibitor were reacted/ml settled Sepharose

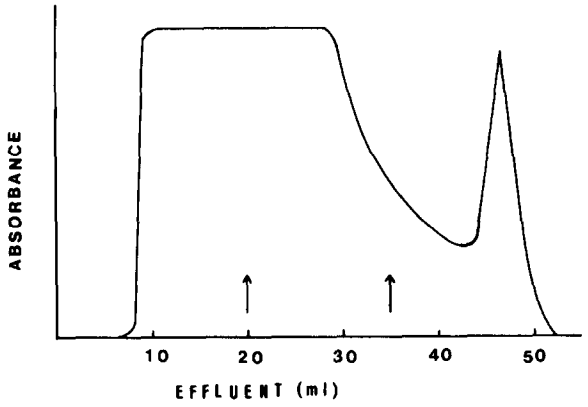

Fig.2. Elution profile of 20 ml crude inhibitor obtained from 1 l urine (see section 2), from a column of inhibitor–Sepharose (1.5 × 4 cm) equilibrated in 0.1 M sodium acetate buffer (pH 5.4). After 20 ml the unadsorbed material was washed out with 15 ml of the same buffer. The bound inhibitor was then eluted with 0.1 M buffer (pH 10). When 4 mg pure inhibitor were chromatographed in the same conditions 90% of the material was bound by the column. Operation temp. 4°C. Arrows indicate changes of buffer.

and free inhibitor was specific. Thus in 0.1 M sodium acetate buffer (pH 5.4) (conditions yielding the highest binding capacity) neither human chorionic gonadotrophin, human serum albumin, ovalbumin and β-lactoglobulin were bound by inhibitor–Sepharose nor glycine–Sepharose and human serum albumin–Sepharose bound free inhibitor.

The elution profile of crude inhibitor on an inhibitor–Sepharose column is illustrated in fig.2. The bulk of activity was found in the peak eluted with the pH 10 buffer and the recovery was ~85% with a 50-fold purification. The inhibitor was still contaminated by some low molecular weight impurities. Material with characteristics (specific antisecretory activity, gel filtration and disc-gel electrophoresis pattern in SDS, amino acid composition) identical to those of the inhibitor prepared using the method in [1] was obtained either after rechromatography on inhibitor–Sepharose or after dialysis. The average yield of pure inhibitor was 2.4 mg/l urine.

The immobilized inhibitor has an high stability at pH 5.4 and 5°C; the binding capacity was practically unchanged after 2 months. Moreover the material can be lyophylized in the presence of 10% sucrose with full maintenance of its properties.

It may be concluded that the use of the 'subunit exchange chromatography' allows to obtain pure inhibitor with a higher yield and in fewer steps than the procedure reported [1]. The method appears promising particularly for isolating material from single individuals in order to correlate physiological or pathological situations to the concentration of the inhibitor in urine.

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