Identification and quantitation of *O*-phosphoserine in human plasma fibronectin

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O-Phosphoserine was positively identified as the phosphorylated moiety in human plasma fibronectin by ^{31}P-NMR of intact peptides These data correlated completely with chemical analyses which demonstrated the presence of *O*-phosphoserine at a concentration of 2 residues/molecule Neither *O*-phosphothreonine nor *O*-phosphotyrosine was detected in partial acid and partial alkaline hydrolysates, respectively

O-*Phosphoserine* *Plasma fibronectin* *Degradation*

1. INTRODUCTION

Fibronectins are multifunctional glycoproteins located in plasma, on cell surfaces and in the extracellular matrix [1–4]. They contain two similar or identical subunits of 220–250 kDa each, held together by disulfide bonds near their COOH terminal domains. Their properties have been well studied, including amino acid composition [5,6], partial amino acid sequence [7,8], carbohydrate composition [9,10], and spectral features [11]. They have been implicated in a wide variety of biological phenomena, e.g., cellular adhesion, cell migration, cytoskeletal organization, differentiation, oncogenic transformation, phagocytosis, and hemostasis [12–14].

It has been shown that fibronectins are phosphorylated [7,15–17]: *O*-phosphoserine [Ser(P)] and *O*-phosphothreonine [Thr(P)] occurring in the protein synthesized by chick embryo fibroblasts [17], whilst only Ser(P) was identified in the proteins synthesized by human fibroblasts and in hamster NIL8 cellular fibronectin [15,16]. Using high-voltage electrophoresis, we have previously demonstrated that only Ser(P) was detectable in human cellular and plasma fibronectin [15]. Furthermore, analysis of the phosphopeptides showed that virtually all of the Ser(P) is located in one specific region of human fibronectin. Recently, we have demonstrated that, with regard to known binding domains of fibronectin, the Ser(P) is localized in the fibrin II domain [18]. Here, we have examined the ^{31}P-NMR spectra from intact peptides of human plasma fibronectin and have quantitated the amount of Ser(P) present in this protein by direct chemical analyses.

2. MATERIALS AND METHODS

2 1. *Isolation of human plasma fibronectin*

Human plasma fibronectin was obtained from The New York Blood Center, Inc. and further purified by sequential gelatin-Sepharose affinity chromatography and heparin-agarose chromatography [19,20]. The purity of the protein was monitored by SDS-PAGE and amino acid analysis.

2.2. *Thermolysin digestion*

400 mg of human plasma fibronectin in 400 ml

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of 20 mM Tris, 0.2 M NaCl, pH 7.5, was digested with thermolysin (Calbiochem) at a substrate to enzyme ratio of 100:1. Incubation was at 30°C for 30 min. At the end of the digestion, 0.5 M EDTA, pH 7.5, was added to a final concentration of 10 mM to stop the reaction and the mixture was chilled to 4°C. It was concentrated to 10 ml by ultrafiltration using an Amicon YM2 filter (M_r cutoff 1000) at 4°C. The retentate was examined by ^{31}P-NMR spectroscopy and for its content of phosphoamino acids.

2.3 *Measurement of the corrected Ser(P) content of human fibronectin*

To measure the true Ser(P) content of fibronectin, it was necessary to correct the values observed in partial acid hydrolysates [21,22] for (i) partial hydrolysis of the protein, (ii) destruction of Ser(P) due to hydrolysis of the phosphate bond after release of Ser(P) from the protein, and (iii) destruction of Ser over the time interval of the hydrolysis. The third correction was necessary since the release of serine was used as a measure of hydrolysis of the protein.

The correction factors were determined as follows: Aliquots of fibronectin in solution (19.5 mg/ml) were hydrolyzed in 4 N HCl at 105°C, for varying times in vacuo. The Ser(P) content of the hydrolysate was measured fluorometrically by HPLC [23]. Amino acid analyses of the partial hydrolysates were carried out on a Beckman 121 M automatic amino acid analyzer. Other aliquots of fibronectin in solution were hydrolyzed in 6 N HCl at 105°C in vacuo for varying periods of time for total amino acid analyses. Other large samples (~25 mg and larger) of fibronectin were partially hydrolyzed in acid [21,22] and alkali [24], respectively, and analyzed for the presence of Thr(P) and Tyr(P) by high-performance liquid chromatography (HPLC) [23]

2.4 *^{31}P-NMR*

^{31}P-NMR experiments were performed on a Nicolet 360 MHz superconducting NMR spectrometer operating at 145.75 MHz and in a magnetic field of 8.45 T. 2048 data point free induction decays (FID) were acquired under conditions where the 45°C phosphorus excitation pulse was provided in 12 μs. The acquisition time per FID was 128 ms, the dwell time was 125 μs which corresponds to a sweep width of 4000 Hz and a resolution limit of 3.9 Hz. A total of 20000, scans were signal-averaged to obtain a single FT spectrum. ^{31}P chemical shifts are recorded as positive downfield from the standard of 50 mM NaH_2PO_4 solution in accord with the IUPAC protocol All spectra were recorded at ambient temperature. The thermolysin-produced peptides were dissolved in 6 M Gdn-HCl and the pH adjusted before and after each ^{31}P-NMR experiment. The range of pH was from 11 to 1, the changes being effected by the addition of NaOH or HCl

3. RESULTS

Fig.1 shows the amount of Ser(P) present in hydrolysates of fibronectin as a function of the time of hydrolysis. Destruction of Ser(P) becomes faster than release after about 4 h hydrolysis. Linear-regression analysis (r = 0.98) produced an ordinate intercept of 1.5 nM/mg protein or 22% more Ser(P) than was found after a 4 h hydrolysis. This correction, designated a, can be found for any time of hydrolysis beyond 4 h if the value obtained is corrected for percent destruction which equals 5.27%/h.

The destruction of Ser during hydrolysis of fibronectin is shown in fig.2. Linear regression analysis (r = 0.95) showed a Ser destruction of 0.18%/h. This is designated as correction factor B. The corrected Ser(P) content was found using the equation:

$$[\text{Ser(P)}]_c = a[\text{Ser(P)}]_o \frac{[(\text{Ser})_{24}(1 + B_{24})]}{[(\text{Ser})_4(1 + B_4)]}$$

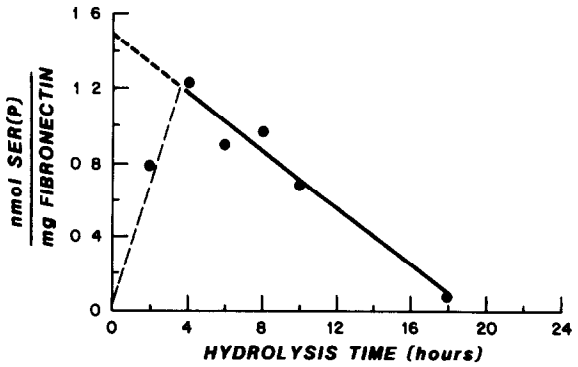

Fig 1. Content of Ser(P) in partial acid hydrolysates (4 N HCl, 105°C) of human fibronectin as a function of time of hydrolysis

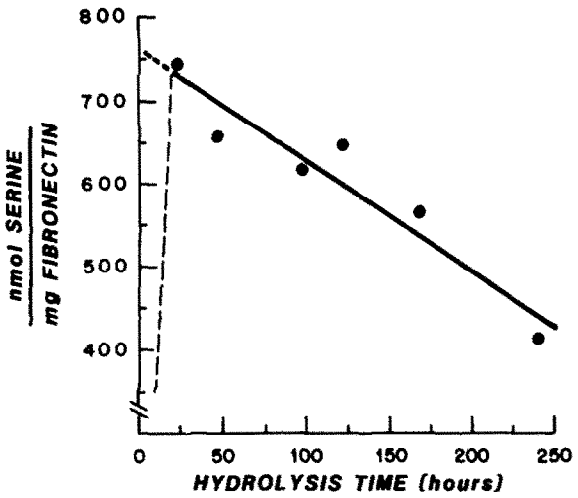

Fig.2 Destruction of Ser during total acid hydrolysis (6 N HCl, 105°C) of human fibronectin as a function of time of hydrolysis

where c and o represent corrected and observed Ser(P) concentration, respectively Rearranging and using the Ser values found at 4 and 24 h of hydrolysis time:

$$\frac{[\text{Ser(P)}]_c}{[\text{Ser(P)}]_o} = 2.12$$

This is the aggregate correction factor for total release of Ser(P) and the breakdown of Ser(P) and Ser during acid hydrolysis. From amino acid analysis of the partial hydrolysates, 0.23 R/1000 of Ser(P) was found for the 4 h hydrolysis. The corrected value, then, is 0 5 R/1000 of Ser(P). The number of Ser(P) residues per molecule of fibronectin is found from

$$\frac{0\ 5 \text{ residues } (440\,000)}{1000 \text{ residues } (109)}$$

where 440 000 is the M_r of fibronectin and the average residue weight from amino acid analysis. From these data, the Ser(P) content of the fibronectin molecule is computed as 2 residues Ser(P)/molecule. No Thr(P) or Tyr(P) was found in the partial acid or alkaline hydrolysates of fibronectin.

3.1 *^{31}P-NMR [25,26]*

A sharp, single resonance was observed in the thermolysin-produced peptides of human fibronectin at pH 9.3 at 520 Hz or 4.8 ppm downfield from H_3PO_4. This chemical shift is characteristic of the orthophosphate of phosphomonoesters such as Ser(P) and Thr(P) and rules out the presence of inorganic orthophosphate, phosphodiesters, symmetrically and unsymmetrically substituted pyrophosphates, polyphosphates and phosphoramidates. Measurement of the pH dependence of the chemical shift when compared with authentic Ser(P) and Thr(P), and β-casein as standards, identified the phosphorylated moiety in fibronectin as Ser(P). Thr(P) could be clearly distinguished from Ser(P), especially at low pH [25].

4. DISCUSSION

As pointed out by Taborsky [27], identification of specific *O*-phosphoamino acids as constituents of proteins after harsh chemical hydrolysis may be at least partly in error due to chemical shifts of the phosphorylated groups from labile to more stable bonds. Therefore identification of the nature of the phosphorylated moiety or moieties by techniques utilizing intact proteins or peptides derived from the proteins is highly desirable. The positive identification of Ser(P) by ^{31}P-NMR in peptides of human plasma fibronectin with no evidence of other phosphorylated components firmly establishes Ser(P) as the single phosphorylated amino acid constituent of human plasma fibronectin. The ^{31}P-NMR data are completely consistent with the results obtained by chemical analyses: failure to detect any Thr(P) or Tyr(P) by a fluorimetric HPLC technique which is capable of detecting very small quantities of these amino acids in the presence of large amounts of Ser(P) (unpublished). Hydrolysis-destruction curves permit us to estimate that 2 residues Ser(P)/molecule are present in human plasma fibronectin.

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