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Conformational changes in human fibrinogen after in vitro phosphorylation and their relation to fibrinogen behaviour

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The far-ultraviolet circular dichroism spectra of fibrinogens phosphorylated by protein kinase C or casein kinase II indicated a conformational change corresponding to an increase in ordered secondary structure. The spectra of protein kinase A- or casein kinase I-phosphorylated fibrinogens did not differ substantially from the control. Fluorescence studies indicated changes in the tertiary structure around tryptophan residues for protein kinase A- or C-phosphorylated fibrinogens, but failed to show any such change for fibrinogen phosphorylated by either of the casein kinases. This latter result was also confirmed by circular dichroism measurements in the near-ultraviolet region. The apparent increase in ordered structure was proposed as an explanation for the slower rate of plasmin degradation seen in fibrinogens after phosphorylation by protein kinase C [6], and casein kinase II, especially as both spectral changes and plasmin degradation rate were unaffected by alkaline phosphatase.

Fibrinogen; Protein phosphorylation; Protein kinase; Circular dichroism; Fluorescence

1. INTRODUCTION

Fibrinogen is a plasma phosphoprotein of molecular mass 340 000 which is made up of six polypeptide chains ($A\alpha_2B\beta_2\gamma_2$) joined by disulphide bonds. It is known that the phosphate content is increased in foetal fibrinogen [1] and after major surgery [2] and that in vitro phosphorylation by protein kinase C causes a decrease in the fibrin fibre thickness in thrombin gelation assays, which is partially reversed by dephosphorylation [3–5]. Moreover, phosphorylation leads to a decreased rate of fibrinogen degradation by plasmin [6] and (Martin, S.C., Forsberg, P.-O. and Eriksson, S.D., unpublished data). Since protein phosphorylation may thus have a physiological function in regulating the properties of fibrinogen, it is of interest to investigate whether phosphorylation of fibrinogen induces any measurable conformational changes in the molecule and if these changes can be related to the functional behaviour of fibrinogen.

2. EXPERIMENTAL

2.1. *Phosphorylations*

Human fibrinogen (F-3879) was obtained from Sigma Chemical (St. Louis, MO, USA) and prepared for use as in [3] before being phosphorylated by protein kinase A, protein kinase C, casein kinase I or casein kinase II. Protein kinase A was purified according to [7], and protein kinase C was purified basically according to [8] with modifications [9]. The casein kinases were both prepared as in [10]. The conditions used for the phosphorylation reactions were basically as described elsewhere [5,11], except that the pH was increased to 8.5 in the protein kinase A reaction to improve the incorporation. After the phosphorylation, the incorporation of phosphate was determined according to [12] and found to be 1.2, 1.25, 3.5 and 4.15 mol phosphate per mol fibrinogen for casein kinase I, casein kinase II, protein kinase C and protein kinase A, respectively. The fibrinogens were chromatographed on a Sephacryl S-300 column to remove the kinases, lyophilised, redissolved in distilled water, dialysed against 30 mM potassium phosphate buffer (pH7.4), containing 150 mM NaCl and stored at −70°C until use. The control fibrinogen was also chromatographed prior to dialysis and storage.

2.2. *Dephosphorylations*

Calf intestinal alkaline phosphatase (Boehringer, Mannheim) was dialysed against 25 mM Tris-HCl buffer (pH 7.4); 125 mM NaCl, and 150 units were used to dephosphorylate 1 mg of the control and phosphorylated fibrinogens. Under the conditions used, approximately half the phosphate was removed from protein kinase A-phosphorylated fibrinogen, 75% from protein kinase C-phosphorylated fibrinogen and 95% from the casein kinase I- or II-phosphorylated fibrinogens.

2.3. *Spectroscopic methods*

Circular dichroism (CD) was measured at 22±2°C with a Jasco J-41A spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Measurements in the far-UV (200–250 nm) region utilised cells with 0.1 cm pathlengths and fibrinogen concentrations of 0.12 mg/ml (0.4 μM). Cells with 1 cm pathlengths and fibrinogen concentrations of 0.7 mg/ml (2.1 μM) were used for measurements in the near-UV (250–320 nm) region. The bandwidth was 2 nm in both wavelength regions. The fibrinogens were dissolved in 30 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl.

Fluorescence measurements were performed at 25°C in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, USA) with fibrinogen concentrations of 0.12 mg/ml (0.4 μM). The excitation

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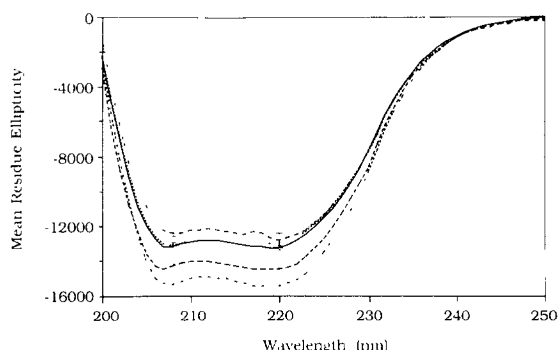

Fig. 1. Far-ultraviolet circular dichroism spectra of control and phosphorylated fibrinogens. Control fibrinogen (——), mean of seven experiments with SEM given at selected wavelengths; protein kinase A-phosphorylated fibrinogen (·····), mean of two experiments with range given at selected wavelengths; protein kinase C-phosphorylated fibrinogen (– – –), mean of four experiments with SEM given at selected wavelengths; casein kinase I-phosphorylated fibrinogen (-----), mean of two experiments with range given at selected wavelengths; casein kinase II-phosphorylated fibrinogen (– –), mean of two experiments with range given at selected wavelengths. The mean residue ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$) was calculated using a mean residue weight of 113 [16].

wavelength was 280 nm and the emission spectrum was measured be tween 300 and 400 nm. The excitation and emission bandwidths were set at 2 and 4 nm, respectively.

Fibrinogen concentrations were measured as in [13].

3. RESULTS

3.1. *Far-ultraviolet circular dichroism*

The far-UV CD spectra of the phosphorylated and control fibrinogens all showed two minima at 208 nm and 220 nm (Fig. 1), in agreement with [14,15]. An increase in negative ellipticity of approx. 10% was observed for the fibrinogens phosphorylated by protein kinase C or casein kinase II. In contrast, phosphorylation by casein kinase I or protein kinase A resulted in negligible changes in ellipticity.

3.2. *Near-ultraviolet fluorescence emission and circular dichroism*

The fluorescence spectra for the various fibrinogens all showed emission maxima at 342 nm (Fig. 2). Protein kinase A phosphorylation gave a 10% decrease in fluorescence, while protein kinase C phosphorylation resulted in a 20% fluorescence increase. However, phosphorylation by either casein kinase I or II did not affect the fluorescence spectra. The latter results were confirmed by near-UV CD studies which showed no differences between control and casein kinase I- or II-phosphorylated fibrinogens.

3.3. *Effects of dephosphorylation*

When the far-UV CD measurements of control and phosphorylated fibrinogens were repeated after treatment with alkaline phoshatase or buffer, no reversibility of the effects of the protein-bound phosphate was seen. The mean residue ellipticities for the dephosphorylated fibrinogens thus varied by less than 3% from the values for the buffer-treated fibrinogens, and none of the values were significantly different from those shown in Fig. 1.

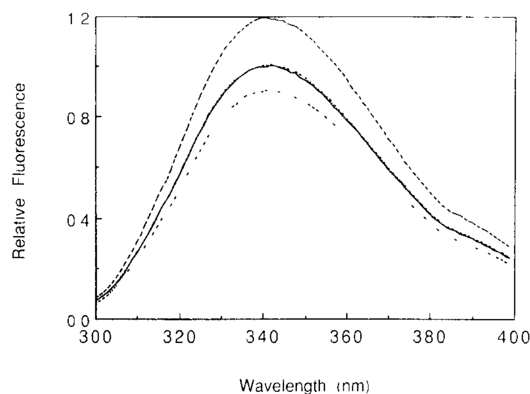

Fig. 2. Fluorescence emission spectra of control and phosphorylated fibrinogens. Symbols as for Fig. 1. All spectra were normalised to a fluorescence intensity for control fibrinogen of 1.0 at the wavelength of the emission maximum. For clarity, the spectrum of casein kinase I-phosphorylated fibrinogen has been excluded, as it did not differ from either the control spectrum or the spectrum of casein kinase II-phosphorylated fibrinogen.

4. DISCUSSION

In vitro phosphorylation of fibrinogen by protein kinase C or casein kinase II resulted in a significant increase in the negative mean residue ellipticity in the far-UV region, indicating a change in the secondary structure of fibrinogen that may involve an increase in α-helix content [17,18]. However, phosphorylation by protein kinase A or casein kinase I resulted in negligible differences from the control, consistent with the phosphate groups added by these enzymes having no appreciable effect on the secondary structure of fibrinogen.

Distinct fluorescence changes were seen after the phosphorylation of fibrinogen by protein kinase A or protein kinase C, while phosphorylation by either of the casein kinases did not alter the fluorescence. These latter results were confirmed by similar CD spectra in the near-UV region, where the aromatic amino acid residues contribute, for fibrinogens phosphorylated by the two casein kinases.

Together, these results indicate that phosphorylation of fibrinogen by protein kinase C results in a conformational change that affects both the secondary structure of the protein and the environment around tryptophan residues. In contrast, the conformational change accompanying the phosphorylation by casein kinase II apparently mainly involves fibrinogen's secondary structure without appreciably altering the tryptophan en-

vironment. Moreover, phosphorylation by protein kinase A seems to result in only local perturbations of fibrinogen's conformation affecting one or more tryptophan residues, but having no appreciable effect on the overall secondary structure. Only phosphorylation by casein kinase I does not lead to a detectable effect on fibrinogen conformation.

The finding that dephosphorylation did not alter the far-UV CD spectra cannot simply be atrributed to incomplete dephosphorylation, as almost all of the ^{32}P-labelled phosphate was removed from the casein kinase-phosphorylated fibrinogens. Analogous non-reversibility has been shown for the inhibition of plasmin digestion after the in vitro phosphorylation of fibrinogen ([6] and Martin, S.C., Forsberg, P.-O. and Eriksson, S.D., unpublished data). In contrast, the effects of fibrinogen phosphorylation on thrombin gelation assays have been shown to be partially reversible [5], with large increases in fibrin fibre thickness after the removal of the endogenous phosphate and a smaller increase in the thickness of the fibrin fibres from protein kinase C-phosphorylated fibrinogen which had been thinner than those of the control.

It has been proposed that plasmin initially attacks the α-chains from the carboxy-terminal ends in a region of disordered structure [19]. Our results suggesting that phosphorylation by two of the four kinases leads to an increase in the ordered structure of fibrinogen are thus consistent with the idea that the action of plasmin is retarded by an increase in the ordered structure in this region of fibrinogen [15]. This appears reasonable since both protein kinase C and casein kinase II phosphorylate sites in the α-chain towards the carboxy-terminus [3,4], and those regions also appear to be phosphorylated by the other two kinases (Martin, S.C., Forsberg, P.-O. and Eriksson, S.D., unpublished data).

The non-reversability of the spectral and plasmin digestion changes suggest that the non-covalent bonds formed after phosphorylation in this new region of ordered structure continue to stabilise the region even after dephosphorylation has removed the phosphate group(s) which caused the initial conformation change.

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