Phosphorylation of human fibrinogen in vitro with calcium-activated, phospholipid-dependent protein kinase and [^{32}P]ATP

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1. INTRODUCTION

Human fibrinogen is a phosphoprotein with part of the phosphorus bound to fibrinopeptide A [1,2]. However, most of the phosphorous remains bound to the fibrin after splitting off the fibrinopeptides [2]. Phosphorylserine occurs at 2 or more locations in the α-chain [3], one of which is Ser_{441} [4]. It was recently found that human fibrinogen is a substrate of cyclic AMP-stimulated protein kinase in vitro with a maximal incorporation of $\geq$ 6 mol phosphate/mol fibrinogen, preferentially into the α-chain [5]. One of the phosphate-accepting sites is Ser_{441} of the α-chain [5].

Most phosphate bound to serine and threonine residues in proteins appears to have regulatory functions and is incorporated in protein kinase reactions. As one approach to study the physiological role of fibrinogen phosphate it was of interest to look for more protein kinases active on fibrinogen. Therefore, extracts from different rat tissues were incubated with [^{32}P]ATP and human fibrinogen. It was found that brain and spleen contain a high fibrinogen kinase activity which was not stimulated by cyclic AMP. After a partial purification from brain tissue it was demonstrated that the main part of the enzyme activity was stimulated by Ca^{2+}, phosphatidylserine and diolein in a similar manner as the protein kinase described in [6]. Some of the methods in [7] were used to obtain a calcium-activated, phospholipid-dependent protein kinase from rat brain. Human fibrinogen is shown to be a good substrate for this protein kinase preparation.

2. EXPERIMENTAL

2.1. *Materials*

[^{32}P]ATP was purchased from New England Nuclear (Boston MA). Human fibrinogen was obtained from Kabi AB (Stockholm). Mixed histone (type II-AS), protamine (salmon sperm), phosvitin, 1,2-diolein and phosphatidylserine were products of Sigma. Sephadex was purchased from Pharmacia Fine Chemicals (Uppsala). The DEAE-cellulose and the phosphorylcellulose used were Whatman DE-52 and P-11, respectively.

2.2. *Enzyme assays*

Protamine kinase and histone kinase activities were assayed as in [8] using 75 μl final incubation vol. Phosvitin kinase activity was estimated under the same conditions. This was also the case for fibrinogen kinase determinations except that 2 mg fibrinogen/ml and 0.1 mM [^{32}P]ATP were used. When the effects of Ca^{2+}, phosphatidylserine and diolein were investigated mercaptoethanol was omitted. Fibrinogen was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) before use. The specific radioactivity of the [^{32}P]ATP was ~ 4×10^4 cpm/nmol. Phosphatidylserine and diolein were dissolved separately in chloroform/methanol (95/5, v/v) to 10 mg/ml and 5 mg/ml, respectively. Phosphatidylserine (15 μl) and diolein (2.4 μl) solutions were emulsified for 15 min at 0°C with 1.98 ml 20 mM Tris–HCl (pH 7.5) with an MSE 150 Watt Ultrasonic Disintegrator. When added to the protein kinase assay, final conc. was 10 μg/ml and 0.8

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μg/ml, respectively. One unit of protein kinase activity was defined as that amount which incorporated 1 pmol phosphate from [^{32}P]ATP to acid-precipitable protein/min under the conditions used.

2.3. *Analytical methods*

One-dimensional polyacrylamide gel electrophoresis was performed using the slab gel system [9] as modified [10]. [^{32}P]Phosphorylserine and [^{32}P]phosphorylthreonine were isolated from acid hydrolysates of ^{32}P-labelled fibrinogen by chromatography on Dowex 50-X8 and Dowex-1-X8 as in [11].

2.4. *Preparation of tissue extracts for estimation of fibrinogen kinase and histone kinase*

Brain, spleen and liver tissue was rapidly removed from decapitated Sprague-Dawley rats (300 g body wt) and homogenized in a Potter–Elvehjem Teflon–glass homogenizer as in [7] with 4 vol. 20 mM Tris–HCl buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA and 10 mM EGTA. Muscle tissue was homogenized with 4 vol. of the same buffer using a Polytron homogenizer. The homogenates were centrifuged at 0–4°C and 80 000 × *g* for 90 min. The supernatants were chromatographed on Sephadex G-50 columns which were equilibrated with 20 mM Tris–HCl buffer (pH 7.5) also containing 1 mM EDTA, 50 mM mercaptoethanol and 10% glycerol. The volume of the samples applied was maximally ~ 15% of the volume of the column. Fractions corresponding to the void volume were pooled and assayed for protein kinase activity with fibrinogen or histone as substrate.

2.5. *Purification of calcium-activated, phospholipid-dependent protein kinase*

Calcium-activated, phospholipid-dependent protein kinase of rat brain was prepared according to the initial steps in [7]. To dissociate the holoenzyme of cyclic AMP-stimulated protein kinase into its catalytic and regulatory subunits, cyclic AMP was added to the supernatant from 12 rat brains (~21 g wet wt) to 10 μl final conc. The supernatant was immediately applied to a 2.2 × 10 cm DE-52 column in equilibrium with 20 mM Tris–HCl (pH 7.5) also containing 10 μM cyclic AMP, 50 mM mercaptoethanol, 5 mM EGTA and 2 mM EDTA. The column was eluted with 500 ml of the starting buffer, followed by a linear gradient of 300 ml starting buffer and 300 ml same buffer, containing 0.4 M NaCl. The eluted fractions were assayed for fibrinogen, protamine, histone and phosvitin kinase activities. Two peaks of fibrinogen kinase activity were obtained (fig. 1). The material of the two peaks were pooled separately and concentrated using Amicon PM 10 filters followed by collodion bags (Sartorius-Membrane Filter, Göttingen). The material from the first fibrinogen kinase peak (pool I) was then chromatographed on a 1.6 × 60 cm Sephadex G-200 column equilibrated with 20 mM Tris–HCl buffer (pH 7.5), 0.5 mM EGTA and 50 mM mercaptoethanol (fig. 2).

The material from the second fibrinogen kinase peak (pool II) was chromatographed on a 5.3 × 44 cm Sephadex G-50 column equilibrated with 20 mM Tris–HCl (pH 7.5) also containing 1 mM EDTA, 50 mM mercaptoethanol and 10% glycerol. The void volume was then applied to a 1.5 × 12 cm phosphocellulose column equilibrated and eluted with the same buffer. Most of the fibrinogen kinase activity was not retarded. The flow-through fraction was then concentrated and chromatographed on a Sephadex G-200 column (fig. 3) as described above for the enzyme from the first fibrinogen kinase peak from the DE-52 column.

3. RESULTS

3.1. *Tissue distribution of fibrinogen kinase activity*

It was shown that extracts from brain and spleen contained a considerable fibrinogen kinase activity in the absence of cyclic AMP while the extracts from liver and muscle had a much lower activity (table 1). When extracts from various tissues were compared, it was found that the fibrinogen kinase activity had a different distribution than the histone kinase activity. This was even more pronounced when the protein kinase activity was tested in the presence of cyclic AMP. While the histone kinase activity varied between different organs within a factor of ~ 2, there was a 30-fold variation between the extracts with regard to fibrinogen kinase activity. From table 1 it is obvious that the extracts from brain and spleen contained fibrinogen kinase activity which was not due to cyclic AMP-stimulated protein kinase. In addition, some of the phosphorylation of fibrinogen was dependent on cyclic AMP-stimulated protein kinase, since cyclic AMP

Table 1

Fibrinogen kinase and histone kinase activities in extracts from different rat organs

	Fibrogen kinase (units/g tissue)		Histone kinase (units/g tissue)	
	− cAMP	+cAmp	− cAMP	+cAMP
Brain	3080	3380	3800	7110
Spleen	1580	1510	1850	5790
Liver	120	110	290	3320
Muscle	280	470	1520	7230

Cell sap, prepared as in section 2, was applied to Sephadex G-50 columns equilibrated with 20 mM Tris–HCl (pH 7.5)–10% glycerol–1 mM EDTA–50 mM mercaptoethanol. Protein kinase activity was assayed as in section 2 with 2 mg fibrinogen/ml or 0.4 mg mixed histone /ml, 0.1 mM [^{32}P]ATP and 5 mM magnesium acetate. Cyclic AMP was 4 μM when present.

stimulated the phosphorylation slightly with an extract from brain and considerably with an extract from muscle.

3.2. *Copurification from rat brain of calcium-activated, phospholipid-dependent protein kinase and fibrinogen kinase*

Brain and spleen, which were shown here to have a high fibrinogen kinase activity (table 1), are also known to contain a large amount of calcium-activated, phospholipid-dependent protein kinase [12,13]. Due to the high activity of this protein kinase in thrombocytes [12] and since calcium and phospholipid are essential in blood coagulation, it seemed to be an interesting possibility that fibrinogen is a substrate of calcium-activated, phospholipid-dependent protein kinase. Therefore, the protein kinase was partially purified from rat brain according to the initial steps in [7], using protamine and histone as substrates. In addition, the fractions were assayed for fibrinogen kinase activity to see whether the kinase activities copurify in the chromatographic systems and whether they have other properties in common.

Fibrinogen kinase activity was eluted from a DE-52 column as one minor and one major broad peak together with protamine and histone kinase activity (fig.1). According to [7], the main protamine kinase peak should represent the calcium-activated, phospholipid-dependent protein kinase [14]. The large phosvitin kinase peak was eluted somewhat later than the second fibrinogen kinase peak. This general pattern was very reproducible. To remove most of the phosvitin kinase activity the material from the second fibrinogen kinase peak was filtered through a phosphorylcellulose column as in section 2. The main part of the fibrinogen kinase activity which was not retained on the phosphorylcellulose column, as well as the material of the first fibrinogen kinase peak from the DE-52 column, were concentrated separately to ~ 2.5 ml and each sample applied to a Sephadex G-200 column.

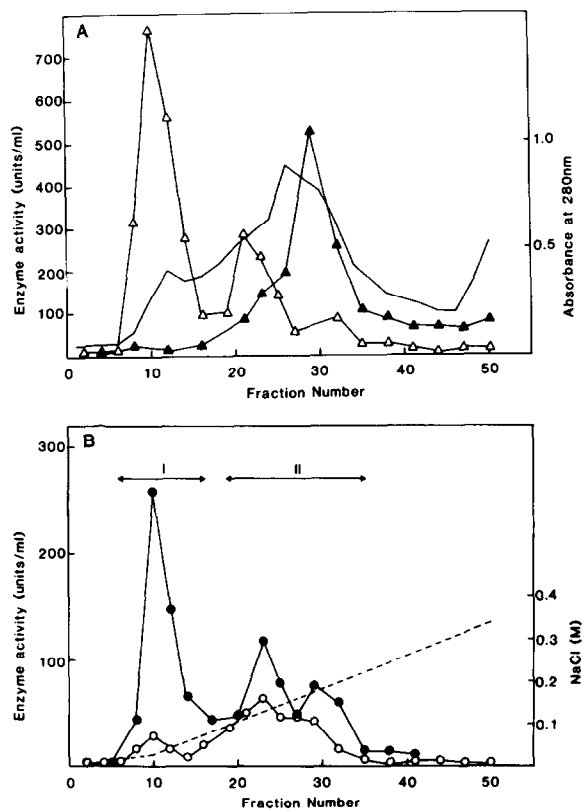

Fig.1. Chromatography of cell sap from rat brain on a DE-52 column: A,B describe the same chromatogram. Protein kinase activity estimated with different substrates as in section 2: (A) (——) absorbance at 280 nm, (△——△) protamine kinase activity, (▲——▲) phosvitin kinase activity; (B) (○——○) fibrinogen kinase activity, (●——●) histone kinase activity, (- - -) [NaCl]; fractions pooled as indicated (I,II).

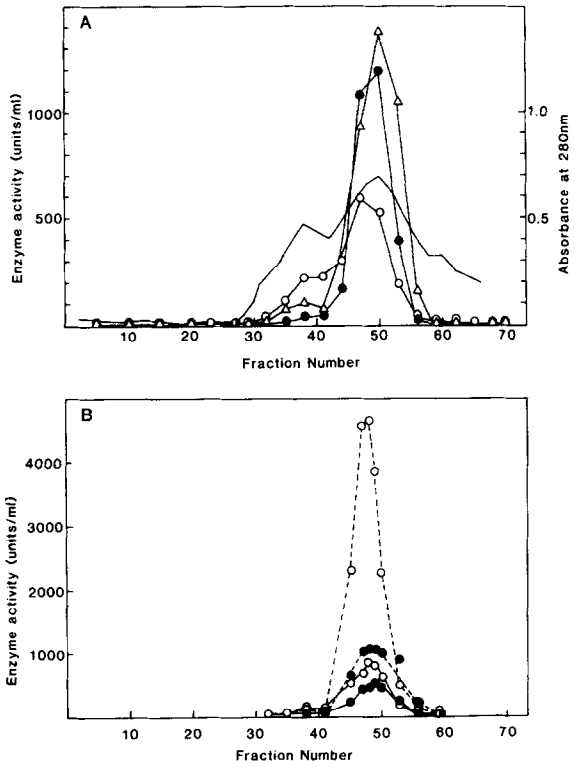

Fig.2. Chromatography of pool I of fibrinogen kinase from DE-52 chromatography in fig.1 on a Sephadex G-200 column, as in section 2: (A) symbols as in fig.1; (B) (○---○), (○——○) fibrinogen kinase activity in the presence and absence, respectively, of Ca^{2+}, phosphatidylserine and diolein; (●---●), (●——●), histone kinase activity in the presence and absence, respectively, of Ca^{2+}, phosphatidylserine and diolein. Ca^{2+} was 0.5 mM when present.

Fig.2A demonstrates a typical Sephadex G-200 chromatogram of material from the first fibrinogen kinase peak from a DE-52 chromatography. The fibrinogen kinase activity was eluted as one main peak preceded by a minor peak. Histone and protamine kinase activity were eluted roughtly in parallel with the fibrinogen kinase activity. The main peak appeared after ~ 0.6 column volume. When the fibrinogen kinase activity was assayed in the presence of 0.5 mM CaCl, phosphatidylserine and diolein there was ~ 5-fold stimulation whereas the activity on mixed histone showed ~ 2-fold stimulation (fig.2B). With other enzyme preparations there was only a 2-fold stimulation of the basal fibrinogen kinase activity and almost no stimulation of the histone kinase activity.

A similar Sephadex G-200 chromatogram was obtained with the material from the second fibrinogen kinase peak eluted from the DE-52 column (fig.3A). While the stimulation of the fibrinogen kinase activity with Ca^{2+} and lipids was considerable, there was hardly any stimulation of the phosphorylation of mixed histone. In some preparations, there was up to 2-fold stimulation of the histone kinase activity.

3.3. *Effect of Ca^{2+}, phosphatidylserine and diolein on the fibrinogen kinase activity*

The fibrinogen kinase peaks eluted from Sephadex G-200 (fig. 2B,3B) were stimulated by a mixture of Ca^{2+}, phosphatidylserine and diolein, indicating that the enzyme activity was of the same type as in [7,14]. To confirm this idea the effect of each lipid component was studied by activity measurements on both enzyme pools with omission of one com-

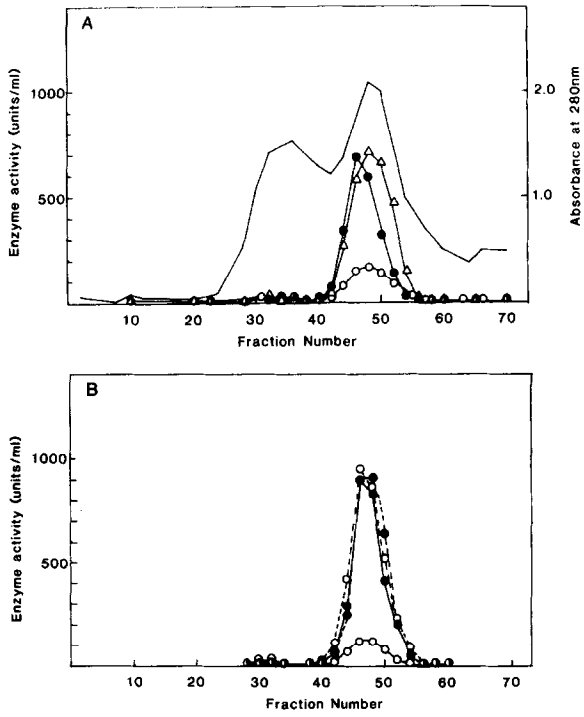

Fig.3. Chromatography of pool II of fibrinogen kinase from DE-52 chromatography in fig.1 on a Sephadex G-200 column, as in section 2: (A),(B) symbols as in fig.2.

Table 2

Effect of Ca^{2+}, phosphatidylserine and diolein on the fibrinogen kinase activity

Incubation conditions	Relative enzyme activity	
	Pool I	Pool II
Control: – Ca^{2+}, – phosphatidylserine, – diolein	100	100
A: 10 μM Ca^{2+}		
Complete	142	344
– phosphatidylserine	102	182
– diolein	120	214
B: 500 μM Ca^{2+}		
Complete	183	627
– phosphatidylserine	96	167
– diolein	179	435

Pools I and II from the DE-52 chromatography were purified on Sephadex G-200 chromatographies (as shown in fig.2 and 3, respectively) and dialyzed against 20 mM Tris–HCl (pH 7.5)–0.03 mM EGTA–50 mM mercaptoethanol before use. The complete assay systems contained 10 μg phosphatidylserine/ml, 0.8 μg diolein/ml and 10 or 500 μM Ca^{2+}.

ponent at a time at two different [Ca^{2+}]. The activity of both enzyme pools was decreased when either phosphatidylserine or diolein was omitted (table 2). The only exception was that diolein did not have any effect on the activity of pool I at 500 μM Ca^{2+}. The activity increased considerably when [Ca^{2+}] was increased from 10–500 μM. Thus, Ca^{2+}, phosphatidylserine and diolein have each been shown to be activators of the fibrinogen kinase activity.

3.4. *Phosphorylation site(s) in fibrinogen*

Since the phosphate of fibrinogen is located to the α-chain [3] one would expect that the enzyme from pools I and II should incorporate labelled phosphate from [^{32}P]ATP into this chain if the enzyme reactions were to have a physiological significance. Therefore, fibrinogen was separately ^{32}P-labelled with both enzyme pools after chromatography on Sephadex G-200 and analyzed by polyacrylamide gel electrophoresis in sodium lauryl sulphate under reducting conditions followed by autoradiography. In both cases nearly all the radioactivity was associated with the α-chains (not shown).

From acid hydrolysates of ^{32}P-labelled fibrinogen, [^{32}P]phosphorylserine and traces of [^{32}P]phosphorylthreonine were isolated by Dowex 50-X8 and Dowex 1-X8 chromatography. Thus, serine residues seem to be preferentially phosphorylated.

4. DISCUSSION

A fibrinogen kinase activity has been found and partially purified from rat brain. The enzyme is stimulated by Ca^{2+}, phosphatidylserine and diolein. It is eluted in a similar position to the protein kinase of Nishizuka on DE-52 and Sephadex G-200 chromatographies. It is heterogenous on these chromatographies, as also described for the enzyme in [7]. The distribution of the fibrinogen kinase between some organs (table 1) is about the same as for the calcium-activated, phospholipid-dependent protein kinase [12,13]. Even though the fibrinogen kinase has not been purified and characterized extensively, it is obvious that it is of the same type as the protein kinase found by Nishizuka's group.

All the phosphorus of fibrinogen seems to be present in the α-chain [3,4]. This subunit is phosphorylated by the cyclic AMP-stimulated protein kinase [5] and, in addition, the protein kinase studied here phosphorylated the α-chain of fibrinogen. It should be pointed out that it is quite usual for a protein to serve as a substrate for $>$ 1 protein kinase. Examples are phosphorylase kinase and glycogen synthase, which are also substrates of the calcium-activated, phospholipid-dependent protein kinase [14].

Due to the essential roles of platelets and fibrinogen in blood coagulation it is interesting that the highest activity of this enzyme has been reported for platelets [12] and that the enzyme might be involved in platelet activation [15].

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