PHOSPHORYLATION OF HUMAN AND RAT BLOOD PLASMA PROTEINS IN VITRO WITH CYCLIC 3′,5′-AMP-STIMULATED PROTEIN KINASE AND [^{32}P]ATP

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1. Introduction

The number of enzymes and other proteins known to be reversibly modified by specific phosphorylation-dephosphorylation reactions is steadily increasing [1]. In several cases this phosphate transfer has been shown to result from hormone stimulation and to influence the activity of key enzymes in mammalian metabolism. Phosphorylation might also be involved in the regulation of other processes, e.g., protein synthesis, nervous conduction and secretion [2]. However, for the majority of the proteins modified in this way present knowledge of the function of the phosphorylation seems to be incomplete.

Hitherto, interest has been focused on intracellular and on membrane phosphoproteins. With the exception of fibrinogen [3,4] we have found no reports on the phosphate content or on any substantial phosphorylation of blood plasma proteins. We have recently found purified human fibrinogen to be a substrate of cyclic AMP-stimulated protein kinase in vitro, although the possible physiological effects of this phosphorylation are as yet unknown [5].

This report describes the phosphorylation of human and rat plasma and serum protein with the catalytic subunit of cyclic AMP-stimulated protein kinase.

2. Experimental

[^{32}P]ATP was a product of New England Nuclear (Boston MA). Sephadex was purchased from Pharmacia Fine Chemicals (Uppsala).

The catalytic subunit of cyclic AMP-stimulated protein kinase from pig muscle was purified as in [6] with some modifications [7]. One unit of protein kinase catalyses the incorporation of 1 pmol [^{32}P]-phosphate/min into mixed histones (7.5 mg/ml) at pH 6.9 and 30°C.

Blood was collected from healthy human adults or from male Sprague-Dawley rats (~300 g body wt) and fed ad libitum on ordinary laboratory chow.

For the preparation of plasma, 4 mM EDTA was used as anticoagulant and the blood samples were centrifuged immediately. Serum was obtained by centrifugation of blood samples after 20 min at room temperature. Phenyl methylsulfonyl fluoride (1 mM final conc.) was added to freshly prepared plasma and serum in order to minimize proteolytic degradation, and serum was made 4 mM with respect to EDTA. The material that was not used immediately was stored frozen and it was only thawed once.

Phosphorylation was performed at 30°C with 0.25 mM [^{32}P]ATP and the indicated amounts of protein kinase in the presence of 10 mM magnesium acetate. The reaction was interrupted by the addition of cold trichloroacetic acid/H_3PO_4 (10% (w/v)/50 mM final conc.). Bovine serum albumin (1 mg/sample) was used as coprecipitant. The precipitate was collected by centrifugation and dissolved in 0.5 ml 0.2 M NaOH. It was washed 4 times by repeated precipitation and dissolution. After the last addition of NaOH the radioactivity was measured as Cerenkov radiation [8]. Samples phosphorylated for electrophoresis were not precipitated (see below).

Prior to agarose gel electrophoresis the phosphorylation was interrupted and excess [^{32}P]ATP removed by passing the incubation mixture through a Sephadex G-50 column equilibrated and eluted with 75 mM barbituric acid–sodium barbiturate buffer (pH 8.6). Electrophoresis in 0.8% (w/v) agarose gel was per-

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formed essentially as in [9]. The protein was stained with Coomassie brilliant blue. When destained, the gel plates were covered with filter paper and left overnight at room temperature. Then the filter papers were removed and the plates were wrapped in thin plastic film and subjected to autoradiography.

Polyacrylamide gel electrophoresis was performed, using the slab gel system in [10]. Prior to application the samples were denatured and reduced in 4 M urea/1% (v/v) β-mercaptoethanol/1% (w/v) sodium dodecyl sulphate (SDS) at 60°C for 60 min. The acrylamide concentration of the runner gel was 11% (w/v). After staining and drying of the gels, autoradiography was carried out.

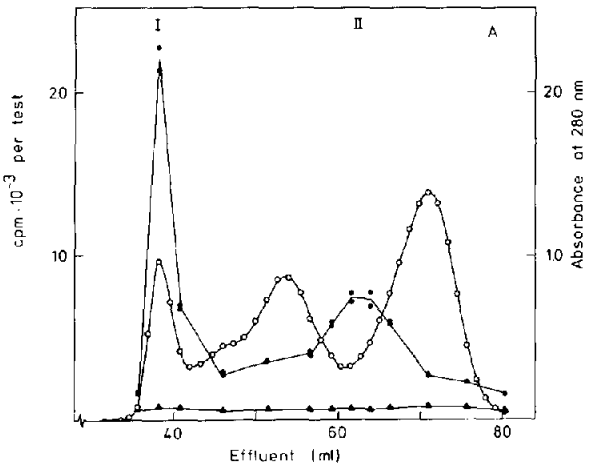
[^{32}P]Phosphorylserine was isolated from acid hydrolysates of [^{32}P]phosphorylated plasma and serum protein by chromatography on Dowex 50-X8 and 1-X8 as in [11].

3. Results

3.1. *G-200 chromatography*

When human and rat plasma or serum was chromatographed on Sephadex G-200, phosphorylatable material was eluted as two main peaks (fig.1). The first peak (I) was eluted with the void volume. The second (II) was eluted at a position corresponding to $M_r \sim$ 100 000. When the chromatography was performed with material that had been incubated with [^{32}P]ATP and protein kinase prior to application, again two [^{32}P]phosphoprotein peaks were obtained, eluting at the same positions as above.

Fig.2 shows the time course of phosphorylation of



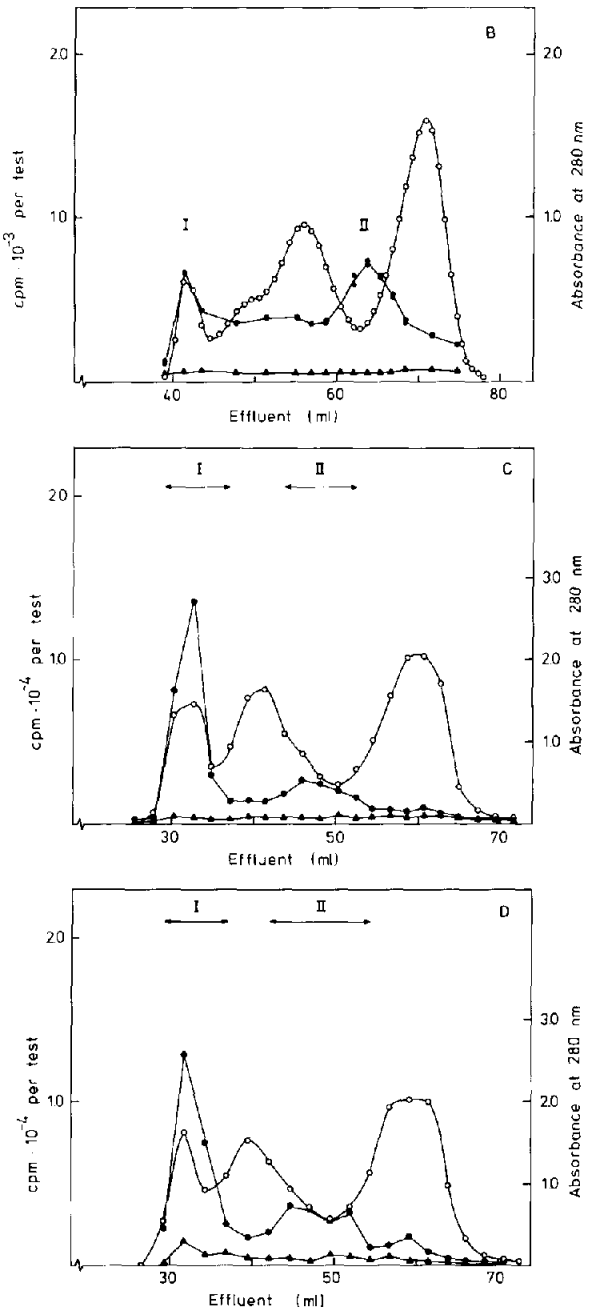


Fig.1. Chromatography of 0.5 ml human plasma (A) and serum (B) on 1.6 × 61 cm columns and chromatography of 0.75 ml rat plasma (C) and serum (D) on 1.8 × 40 cm columns of Sephadex G-200. The columns were equilibrated and eluted with 10 mM potassium phosphate buffer/1 mM EDTA (pH 7.5): A_{280} (○——○); ^{32}P radioactivity incorporated into 100 μl samples from separate fractions on incubation for 20 min (A,B) or 25 min (C,D) with [^{32}P]ATP (57 000 cpm/nmol) in the absence (▲——▲) or presence (●——●) of protein kinase (1000 units/test in A,B; 600 units/test in C.D).

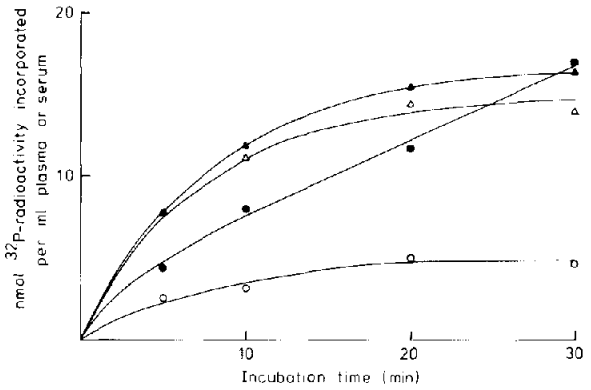


Fig.2. [^{32}P]Phosphate incorporation into peak I and peak II material from human plasma and serum. Each point represents the mean of two determinations. The incubation mixtures contained 200 μl pooled material from the G-200 chromatography and 2000 units protein kinase. Plasma peak I (●——●), plasma peak II (▲——▲), serum peak I (○——○), serum peak II (△——△).

material from peak I and peak II from human plasma and serum. No plateau value was obtained for fraction I from plasma. The phosphate incorporation into fraction I from serum was markedly lower and followed another time course. The [^{32}P]phosphate incorporation into material from peak I and peak II from rat plasma and serum under similar conditions amounted to higher values, corresponding to ~20 nmol and 10 nmol [^{32}P]phosphate/ml plasma or serum in a 30 min incubation period for peak I and II, respectively. Thus, in this case no significant difference between plasma and serum was detected regarding the amount of [^{32}P]phosphate incorporated into fraction I. This is consistent with the results depicted in fig.1C,D.

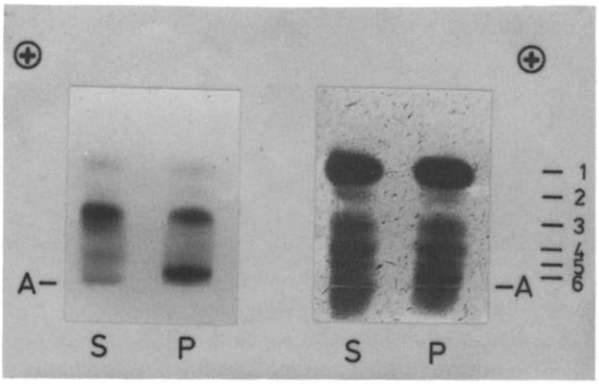


Fig.3. Agarose gel electrophoresis of human serum (S) and plasma (P) after incubation with [^{32}P]ATP and protein kinase. Autoradiogram (left) and protein pattern (right). The positions of the application slit (A) and of the albumin (1), α_1 (2), α_2 (3), β_1 (4), β_2 (5) and fibrinogen (6) bands are indicated in the figure.

3.2. *Agarose gel electrophoresis*

When human plasma was subjected to agarose gel electrophoresis after incubation with [^{32}P]ATP and protein kinase the radioactivity appeared in two main regions, coinciding with the α_2- and the fibrinogen-band, respectively. In serum treated in the same way, the radioactivity was found corresponding to the α_2-band (fig.3). Plasma peak I was found to be

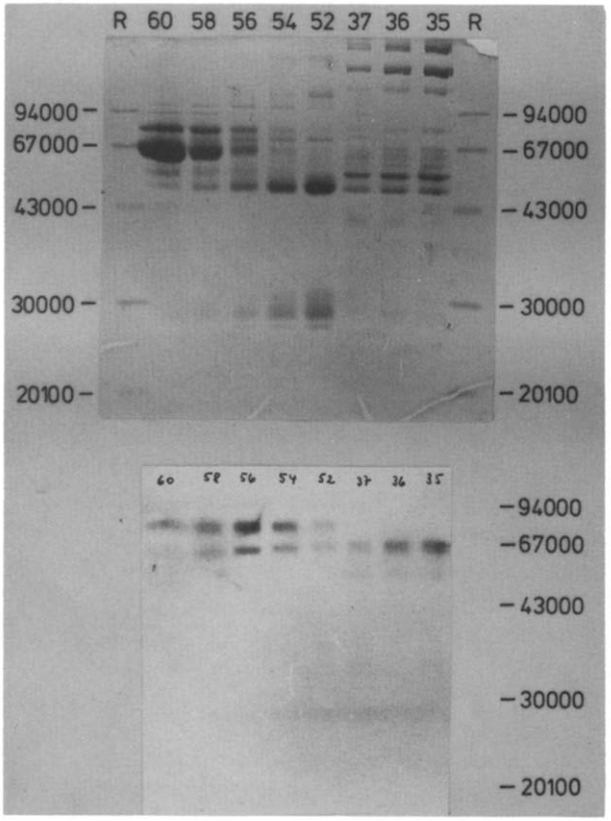


Fig.4. SDS–Polyacrylamide gel electrophoresis under reducing conditions of samples from a Sephadex G-200 chromatography of human plasma, incubated with [^{32}P]ATP and protein kinase prior to application on the column. Protein pattern (above) and autoradiogram (below). The M_r-values of the reference proteins (R) are indicated in the figure. The figures 35–60 indicate fraction numbers, where 35 represents a fraction in the middle of peak I and 56 represents a fraction in the middle of peak II (see fig.1A).

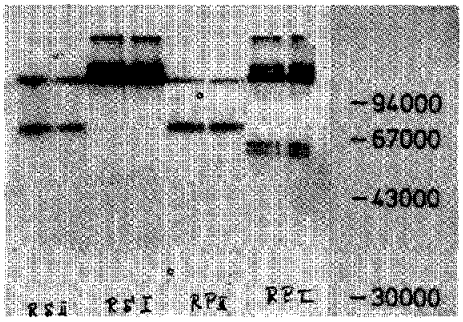


Fig.5. SDS–Polyacrylamide gel electrophoresis under reducing conditions of samples from rat peak I and peak II (fig.1). Rat plasma peak I (RPI), rat plasma peak II (RPII), rat serum peak I (RSI) and rat serum peak II (RSII). Autoradiogram.

the source of the radioactivity coinciding with the fribrinogen band and peak II was the source of the radioactive material migrating with the α_2-fraction.

On separation of rat plasma proteins by agarose gel electrophoresis after phosphorylation, the radioactivity appeared in a dominating fraction coinciding with the albumin band and in a minor band just anodal to the application slit. The latter component was not found in serum.

3.3. *SDS–polyacrylamide gel electrophoresis*

The [^{32}P]phosphoprotein of peak II from human plasma or serum was resolved into two components by SDS–polyacrylamide gel electrophoresis under reducing conditions (fig.4). The M_r-values were estimated to be 80 000 and 67 000, respectively. Only one phosphorylated component, with est. M_r 67 000, was detected by this method in plasma peak I. Due to the low degree of phosphorylation peak I from serum was not investigated in this respect.

The [^{32}P]phosphoprotein of peak II from rat plasma or serum appeared on the polyacrylamide gels as a component with $M_r \sim 80\,000$ (fig.5). In addition there was a component with $M_r > 100\,000$. The ^{32}P-radioactivity of peak I appeared in the high M_r region, except for 2 minor bands derived from plasma and corresponding to $M_r \sim 60\,000$ and 65 000.

3.4. *Isolation of [^{32}P]phosphorylserine*

From acid hydrolysates of [^{32}P]phosphorylated peak I and peak II material from human or rat plasma, [^{32}P]phosphorylserine but no [^{32}P]phosphorylthreonine could be isolated.

4. Discussion

The data obtained indicated that fibrinogen was the main phosphorylatable component of fraction I from human blood under the conditions used, since the [^{32}P]phosphoprotein was eluted from Sephadex G-200 at the same position as is fibrinogen, it comigrated with the fibrinogen band on agarose gel electrophoresis and the M_r as estimated by polyacrylamide gel electrophoresis in detergent under reducing conditions was 67 000, similar to the M_r of the α-chain of human fibrinogen. When purified human fibrinogen is phosphorylated with cyclic AMP-stimulated protein kinase, the phosphate is incorporated primarily into the α-chain [5]. Furthermore, the phosphorylation of purified human fibrinogen follows a time course similar to that obtained for fraction I from human plasma. In addition, the phosphate incorporation into fraction I from serum was much lower.

Rat fibrinogen, however, did not seem to be a major phosphorylatable component in our experiments. The phosphate incorporation into fraction I was not significantly lower in serum than in plasma and only a minor fraction of the phosphoprotein seemed to migrate with fibrinogen on agarose gel electrophoresis.

The identity of the phosphorylatable components of fraction I from rat blood and of fraction II from rat or human blood is not yet clear. Caution in the interpretation of electrophoretic mobility in the agarose gels is needed, since the degree of phosphorylation might influence the migration of the protein.

For proteins phosphorylated stoichiometrically at a significant rate it is reasonable to assume that the phosphorylation might be of physiological importance. The phosphorylation of plasma and serum proteins was relatively rapid. Only a limited number of proteins were phosphorylated (fig.3–5) and the amount of phosphate incorporated was fairly large. This is suggestive of a possible physiological role of the phosphorylation. Interesting in this context might be the stimulatory effects of stress and different hormones on blood coagulation and fibrinolysis [12]. The blank values obtained on incubation of plasma and serum with ATP and Mg^{2+} in the absence of added protein kinase were low and did not increase in the presence of cyclic AMP (10 μM). This indicated that the source of the protein kinase that might be operative in vivo in the phosphorylation of plasma proteins should be somewhere else, e.g., in the liver or in the formed elements of blood.

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References

[1] Krebs, E. G. and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923–959.
[2] Weller, M. (1979) in: Protein Phosphorylation, Pion, London.
[3] Blombäck, B., Blombäck, M., Edman, P. and Hessel, B. (1962) Nature 193, 883–884.
[4] Blombäck, B., Blombäck, M. and Searle, J. (1963) Biochim. Biophys. Acta 74, 148–151.
[5] Engström, L., Edlund, B., Ragnarsson, U., Dahlqvist-Edberg, U. and Humble, E. (1980) Biochem. Biophys. Res. Commun. 96, 1503–1507.
[6] Bechtel, P. J., Beavo, J. A. and Krebs, E. G. (1977) J. Biol. Chem. 252, 2691–2697.
[7] Humble, E., Dahlqvist-Edberg, U., Ekman, P., Netzel, E., Ragnarsson, U. and Engström, L. (1979) Biochem. Biophys. Res. Commun. 90, 1064–1072.
[8] Mårdh, S. (1975) Anal. Biochem. 63, 1–4.
[9] Johansson, B. G. (1972) Scand. J. Clin. Lab. Invest. 29/suppl. 124, 7–19.
[10] O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021.
[11] Ljungström, O. and Engström, L. (1974) Biochim. Biophys. Acta 336, 140–150.
[12] Seyle, H. (1976) in: Stress in Health and Disease, pp. 705–711, Butterworths, Boston, London.