PHOSPHORYLATION OF PROTEINS EXTRACTED FROM HUMAN ERYTHROCYTE MEMBRANE

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1. Introduction

Human and animal erythrocyte membrane contains protein kinase activities able to phosphorylate endogenous substrates from [γ-^{32}P]ATP in presence and in absence of cyclic nucleotides. [1–8]. The sum of these activities may be assayed in whole ghosts suspensions and the stimulation by cyclic nucleotides determined by adding such nucleotides in the assay mixture. However ghost phosphorylation is influenced by the structural arrangement of membrane proteins which controls the accessibility of the sites for phosphoryl donor, cyclic nucleotides and phosphorus acceptance. The steric relations, between the subunits of enzyme molecules and the protein substrates, order and limit the phosphorylating activities included in the intact membranes. So assayed, phosphorylation of total membrane does not reflect the potential activity of the enzymes. Moreover the characteristics of enzyme kinetics and the definition of substrates may be different in the total membrane and in solubilized proteins. We have undertaken to study the protein kinase activities and their substrates in various protein fractions, extracted from total membranes. We present herein the results obtained from proteins solubilized at low ionic strength and alkaline pH.

2. Materials and methods

Fresh heparinized blood from normal subjects was centrifuged and the buffy coat was removed. The erythrocyte was washed three times in phosphate buffered (phosphate buffer pH 8, 0.005 M) isotonic saline.

Ghosts were prepared following Dodge et al. [9]. For the preparation of hydrosoluble proteins, the white pellet was transferred into a dialysis bag and dialyzed overnight against distilled water made alkaline to pH 9 by NH_4OH + β-mercaptoethanol (BME) 1 mM + EDTA 1 mM. Ghosts suspension was then centrifugated at 100 000 × *g* in a Beckman preparative ultracentrifuge and the supernatant containing the hydrosoluble proteins was collected (fraction I).

Ammonium sulfate precipitation was performed from fraction I at 50% $(NH_4)_2SO_4$ saturation for 2 h at 4°C. The precipitate collected by centrifugation at 3000 × *g* was dissolved in Tris–Cl buffer, pH 8.5, 0.025 M + BME 5 mM + EDTA 1 mM + NaCl 50 mM (buffer A) and dialyzed overnight against the same buffer to remove ammonium sulfate (fraction II). The supernatant was kept frozen at –70°C until utilization (fraction III).

An acidic precipitation was performed from fraction II by slowly adding acetate buffer 0.5 M, pH 5.3. After centrifugation at 3000 × *g* the precipitate, mainly composed of spectrin, was solubilized by dropwise addition of Tris 0.1 M, pH 10.5, then dialyzed overnight against the above Tris–Cl buffer (buffer A) (fraction IV). The supernatant was dialyzed in the same way and proteins were precipitated again by ammonium–sulfate (50% saturation) (fraction V). Before use fraction V was dissolved in buffer A and dialyzed overnight against this buffer.

Spectrin was purified from fraction IV by gel filtration in a column of Ultrogel AcA 34 (100 × 2.5 cm) previously equilibrated with buffer A. It was then concentrated by ammonium sulfate precipitation and kept as precipitate at –70°C.

Autophosphorylation of various fractions by [γ-^{32}P]ATP was performed at pH 6 and pH 7.4 following Guthrow et al. [2] as elsewhere reported with details [10]. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed following Fairbanks et al. [11] using gels 12 cm long. Gels were stained with Coomassie Blue. After staining, the peptide bands were cut up; the gel-discs were dissolved in H_2O_2 at 80°C for 4 h, then mixed with 10 ml of scintillation fluid; incorporated radioactivity was determined by liquid scintillation spectrometry (Intertechnic ABAC SL 40).

Results of phosphorylation assays were expressed as pmol ^{32}P transferred from [γ-^{32}P]ATP/mg protein in 5 min, unless otherwise indicated. Peptide labelling in polyacrylamide gels was expressed in cpm with correction for spontaneous decrease of ^{32}P radioactivity and for a constant level of membrane proteins poured on top of gels (50 μg).

[γ-^{32}P]ATP (spec. act. 2–4 Ci/mmol) was purchased from Radiochemical Centre (Amersham); 3′5′-cyclic AMP from Boehringer (Mannheim) acrylamide and bis-acrylamide from Eastman-Kodak and other chemicals from Merck.

3. Results

3.1. *Autophosphorylation of total hydrosoluble proteins (fraction I)*

At pH 6 hydrosoluble protein phosphorylation without cAMP addition was lower than the phosphorylation of total membrane in the same conditions; however stimulation by cAMP was much more important than it was in total membrane : incorporation of ^{32}P/mg protein was increased by 4–6-fold when cAMP was added (fig.1).

With Tris–Cl buffer, pH 7.4, autophosphorylation in absence of cAMP was slightly higher than the phosphorylation, at pH 6; with cAMP added it was slightly lower than at pH 6.0.

Analysis of gels after phosphorylation without cAMP addition showed that the main substrates were band O and bands I and II of the spectrin. Very low labelling was observed in bands II_4, III and VI. With cAMP addition, phosphorylation was increased by 3-fold in bands O, I, II, II_4, III and VI; highly labelled bands were now observed corresponding to the components II_1 and V (fig.2).

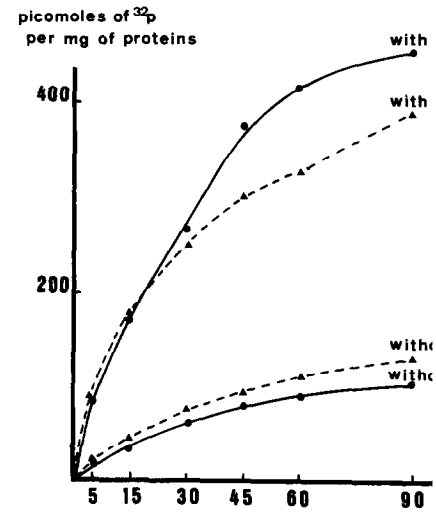

Fig.1. Total phosphorylation of hydrosoluble m protein with respect to the incubation time at p and 7.4 (▲- - -▲) without and with cAMP additi

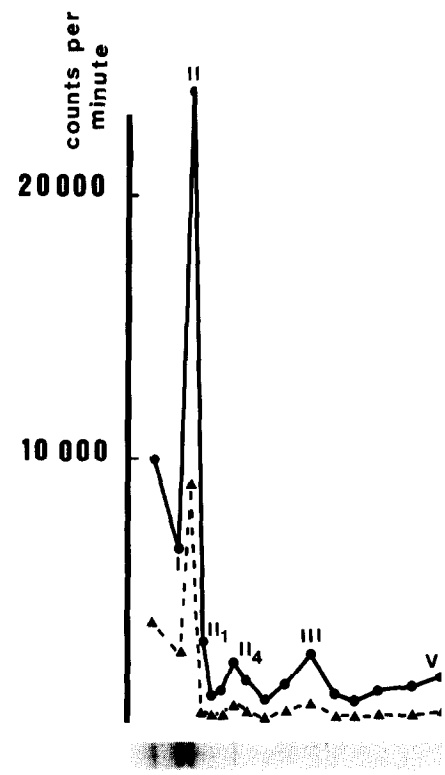

Fig.2. Radioactivity incorporated in the hydroso brane proteins (fraction I) separated by SDS–PA incubation with [γ-^{32}P]ATP, at pH 6, without (• with (•——•) cAMP addition.

3.2. *Fractionation of phosphorylated hydrosoluble proteins*

In order to precisely determine the nature and number of the phosphorylated peptides we have performed phosphorylation of 6.5 mg hydrosolubles proteins with cAMP and 6.5 mg without cAMP. Concentrations of ATP, Mg^{2+} and cAMP were the same as in the standard assay mixture. Phosphorylation was carried out at pH 6 since the above results had demonstrated that cAMP-dependent protein kinase activity was greater at this pH than at pH 7.4. Phosphorylated hydrosoluble proteins were then fractionated as indicated in Materials and methods. Nearly all of the incorporated radioactivity was included in the ammonium sulfate precipitate (fraction II).

The acidic precipitate (fraction IV) contained about 80% of the radioactivity incorporated in the fraction II. Total incorporation of ^{32}P was about 5-fold higher in presence of cAMP than it was without AMP.

Analysis of peptides by SDS–PAGE showed that fraction IV main component was spectrin, the two components of which were labelled without cyclic AMP; however radioactivity incorporated in component II was 6-fold that of component I. The phosphorylation was stimulated 3.7-fold in component I and 4.7-fold in component II in presence of cAMP. Minor bands poorly labelled in absence of cAMP were important substrates for cAMP-dependent protein kinase. Such bands were difficult to identify following the Fairbank's nomenclature. Several ones were located between bands 2 and 3: incorporated radioactivity was increased by 5–7-fold in presence of cAMP. In one of the components of complex III radioactivity was increased by 12-fold in presence of cAMP. A peptide located between band IV and V was phosphorylated 10-fold more in presence of cAMP; two other peptides: one responding to band V and the other one to band VI were phosphorylated, respectively, 14- and 18-fold more in presence of cAMP than without (fig.3).

The supernatant of acidic precipitation (fraction V) contained about 15% of the radioactivity incorporated in fraction II. SDS–PAGE patterns were characterized by the presence of traces of spectrin and other components, and a predominance of low molecular weight peptides. Many bands were poorly phosphorylated in the absence of cAMP; however some peptides are substrates for cAMP-dependent protein kinase activity:

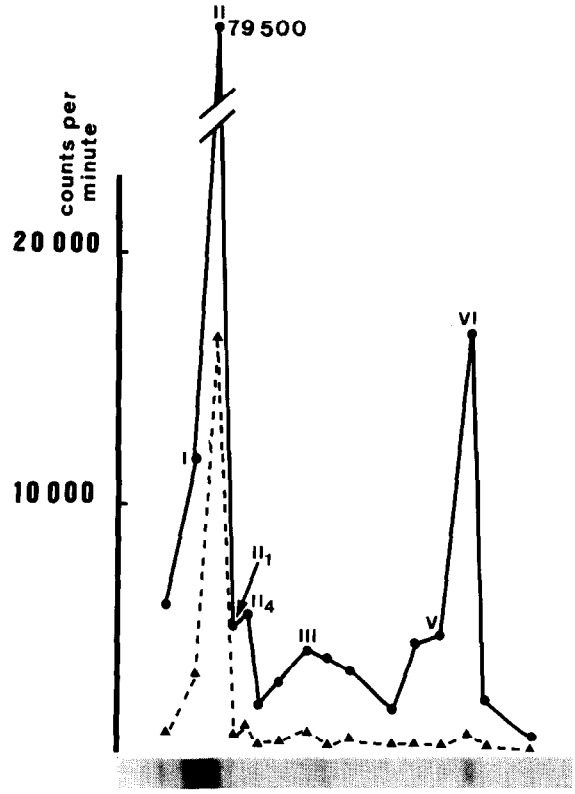

Fig.3. Radioactivity incorporated in the peptides of acidic precipitate (fraction IV) separated by SDS–PAGE after phosphorylation of total hydrosoluble proteins with [γ-^{32}P]ATP, at pH 6, without (▲- - -▲) and with (●——●) cAMP addition.

radioactivity incorporated with cAMP added was increased by 14-fold in the minor band II_8, by 18-fold in a band of complex III and, respectively, by 20, 30- and 36-fold in three bands corresponding to unindentified low molecular weight peptides (fig.4).

3.3. *Autophosphorylation of fractionated hydrosoluble membrane proteins*

Assayed in the standard conditions at pH 6, autophosphorylation occurred in fraction II (ammonium sulfate precipitate) and fraction V (supernatant of the acidic precipitation). It was almost completely lacking in fraction III (supernatant of ammonium sulfate precipitation) and fraction IV (acidic precipitate). Purified spectrin was devoid of protein kinase activity with or without cAMP.

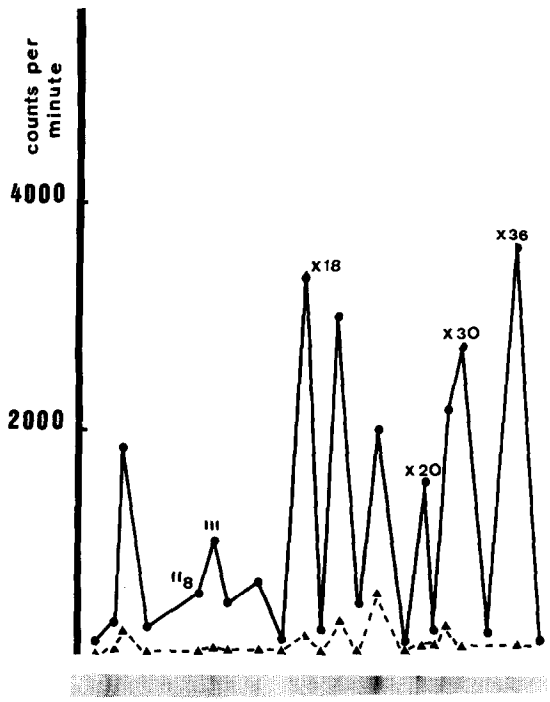

Fig.4. Radioactivity incorporated in the peptides of the supernatant of acidic precipitate (fraction V) separated by SDS–PAGE after phosphorylation of total hydrosoluble proteins with [γ-^{32}P]ATP, at pH 6, without (▲- - - -▲) and with (●——●) cAMP addition.

4. Discussion

Autophosphorylation of hydrosoluble proteins is much more important than that of total membrane and strikingly dependent upon the cAMP; kinetic of the reaction is very different from that of the total membrane phosphorylation. This result indicates that in the standard conditions used for total membrane phosphorylation assay, only one low part of the cAMP-dependent protein kinase activity is available and that the holoenzyme of a cAMP-dependent membrane protein kinase is present in the hydrosoluble proteins. Fractionation of these hydrosoluble proteins shows that the enzyme activity is not related to the purified spectrin. To date we do not know whether activity obtained with such procedure is or is not identical to that extracted with the triton–borate system [12] which solubilized preferentially complex III of erythrocyte membrane [13].

The presence of a highly active cAMP-dependent protein kinase in total hydrosoluble proteins and in their fractionated components makes the study of substrates more accurate in it than in total membrane. As others, we have found that in total membrane only component II of the spectrin was phosphorylated and that its phosphorylation increased poorly in presence of cAMP; in the solubilized proteins both components of the spectrin doublet are phosphorylated; the incorporated radioactivity is more important in the second component but it was increased by about 4-fold in both when cAMP was added to the reaction mixture. So in total membrane only one part of the spectrin may be attained by phosphorylating activities.

As demonstrated in total hydrosoluble proteins and in the various fraction, several peptides bands located between components II and III are phosphorylated by cAMP-dependent protein kinase: components II_1, II_4 and a peptide close to component III being probably II_8. The principal transmembrane protein of complex III is not extracted with hydrosoluble proteins: however one peptide of its zone is also phosphorylated by cAMP-dependent protein kinase. Another peptide responding to band V is labelled: we do not know if really it is actin. Furthermore the bands most actively phosphorylated in presence of cAMP are low molecular weight peptides located beyond component VI towards the anode; it is worthy to note that these peptides are present as minor bands after SDS–PAGE of total membrane proteins solubilized with 1% SDS but they are not yet identified.

4. Conclusions

This study of the autophosphorylation of membrane 'hydrosoluble' proteins allows the following conclusions:

(1) Total membrane phosphorylation assay does not reflect the potential activity of the membrane bound cAMP-dependent protein kinase.
(2) In total membrane only one part of the spectrin is phosphorylated.
(3) cAMP-Dependent protein kinase activity is extracted from the membrane with the hydrosoluble proteins.
(4) Several minor peptides of low molecular weight are highly specific substrates for cAMP-dependent protein kinase.

References

[1] Rubin, C. S., Erlichman, J. and Rosen, O. (1972) J. Biol. Chem. 247, 6135–6139.
[2] Guthrow, C. E., Allen, J. F. and Rasmussen, H. (1972) J. Biol. Chem. 247, 8145–8153.
[3] Roses, A. D. and Appel, S. H. (1973) J. Biol. Chem. 248, 1408–1411.
[4] Tao, M. and Hackett, P. (1973) J. Biol. Chem. 248, 5324–5332.
[5] Avruch, J. and Fairbanks, G. (1974) Biochemistry 13, 5507–5513.
[6] Fairbanks, G. and Avruch, J. (1974) Biochemistry 13, 5514–5521.
[7] Traugh, J. A. and Traut, R. R. (1974) J. Biol. Chem. 249, 1207–1212.
[8] Hosey, M. M. and Tao, M. (1976) Biochemistry 15, 1561–1568.
[9] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[10] Boivin, P. and Galand, C. (1977) in press.
[11] Fairbanks, G., Steck, T. L. and Wallach, D. E. H. (1971) Biochemistry 10, 2506–2617.
[12] Rubin, C. S. (1975) J. Biol. Chem. 250, 9044–9052.
[13] Yu, J., Fischman, D. A. and Steck, T. L. (1973) J. Supramol. Struc. 1, 233–238.