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INTERRELATIONSHIPS BETWEEN PROTEIN KINASES AND SPECTRIN PHOSPHORYLATION IN HUMAN ERYTHROCYTES

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Summary

Casein kinase and histone kinase(s) are solubilized from human erythrocyte membranes by buffered ionic solutions (0.1 mM EDTA and subsequent 0.8 M NaCl, pH 8) containing 0.2% Triton X-100.

Casein kinase is separated from histone kinase(s) by submitting the crude extracts directly to chromatography on a phosphocellulose column, eluted with a continuous linear gradient of potassium phosphate buffer, pH 7.0, containing 0.2% Triton X-100.

Under these conditions, the membrane-bound casein kinase activity is almost completely recovered into a quite stable preparation, free of histone kinase activity. In contrast, it undergoes a dramatic loss of activity when the extraction and the subsequent phosphocellulose chromatography are carried out with buffers which do not contain Triton X-100.

Isolated spectrin, the most abundant membrane protein, is phosphorylated, in the presence of [γ-^{32}P]ATP, only by casein kinase while histone kinase is ineffective.

Only the smaller subunit (band II) of isolated spectrin (and not the larger one (band I)) is involved in such a phosphorylation process, as in the endogenous phosphorylation occurring in intact erythrocytes.

Introduction

A number of studies have shown that the membrane proteins of human erythrocytes are phosphorylated by the endogenous membrane-bound protein kinases (cyclic AMP-dependent and -independent). Moreover, it has been shown by the results of this [1] and other laboratories [2] that the membrane proteins can also be phosphorylated by protein kinases located in the cytosol.

However, the relationships between these multiple protein kinases and their membrane-protein substrates need to be better defined.

In particular, in the past few years, increasing interest has been devoted to the phosphorylation of spectrin, the most abundant membrane protein located on the cytosolic surface, since its phosphorylation state, modulating the interaction with actin and other membrane components, is believed to control the shape, deformability and functional properties of membranes.

At present, however, conflicting findings are reported in the literature [3—7] dealing with the question as to whether spectrin is phosphorylated by one or more protein kinases and as to whether only the smaller (band II) or both the spectrin subunits are involved in the phosphorylation process.

The present work, carried out with the aim of providing better insight in this unsettled matter, shows that also in solution as in intact erythrocytes, spectrin can be phosphorylated only on its smaller subunit (band II) and that membrane-bound casein kinase (but not histone kinase) is involved in such a phosphorylation process.*

Methods and Materials

Preparation of erythrocyte ghosts. Outdated human blood was donated by the Hospital blood bank (Padua) in sterile bottles containing acid/citrate/dextrose (anticoagulant). Hemoglobin-free red cell ghosts were prepared according to the procedure of Dodge et al. [8] at pH 8, except that all solutions contained 0.05 mM phenylmethylsulfonyl fluoride (PMSF).

Solubilization and separation of spectrin from membrane-bound protein kinases. White erythrocyte membranes (5—6 mg/ml) were extracted with 0.1 mM EDTA, pH 8, containing 0.05 mM PMSF for 20 min at 37°C by stirring, and recovered by centrifugation at 105 000 × *g* ('EDTA extract'). EDTA extraction was repeated once more under the same conditions. Subsequently, the pellet was extracted with 0.8 M NaCl, pH 8, containing 0.05 mM PMSF for 12 h at 4°C ('0.8 M NaCl extract') and recovered by centrifugation at 105 000 × *g*. The pellet ('extracted membrane') was resuspended in 15 mosM phosphate buffer (pH 8) to a final concentration of 5—6 mg/ml. The two EDTA extracts, pooled together, and the subsequent 0.8 M NaCl extract were separately concentrated by ultrafiltration through a Diaflo membrane. The EDTA and 0.8 M NaCl extracts, after concentration, were dialyzed overnight against buffer A (0.1 M Tris-HCl, pH 7.5, 5 mM EDTA, 0.05 mM PMSF containing 0.5 M NaCl) and then separately subjected to gel filtration through a Sepharose 6B column (1.8 × 120 cm) equilibrated with buffer A (4-ml fractions, flow rate 6 ml/h). In other experiments, the two extracts were dialyzed overnight against buffer B (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.05 mM PMSF containing 0.1 M NaCl) and then subjected to filtration through the same Sepharose 6B column equilibrated with buffer B. Sepharose fractions were tested for the presence of spectrin by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

* Preliminary accounts of this work have been presented at the 5th Italian Congress of Biochemistry, Lucca (1979).

according to the procedure of Fairbanks et al. [9] and for protein kinase activities by following the procedure described below.

The fraction containing spectrin were pooled together, concentrated by filtration through a Diaflo membrane and dialyzed overnight against buffer C (5 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1 mM mercaptoethanol) to remove NaCl.

Solubilization and purification of membrane-bound protein kinases. White erythrocyte membranes (450 mg protein) were extracted with 90 ml of 10 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 0.05 mM PMSF and 0.2% (w/v) Triton X-100, by stirring for 4 h at 0°C and then recovered by centrifugation at 105 000 × *g*. After the removal of the supernatant (referred to as 'EDTA-Triton' extract), the pellet was extracted with 90 ml of buffer D (10 mM potassium phosphate, pH 7.0, 4 mM mercaptoethanol and 0.2% Triton X-100) containing 0.8 M NaCl. After stirring at 0°C for 4 h, the supernatant (referred to as 'NaCl-Triton' extract) was removed by centrifugation at 105 000 × *g*. The EDTA-Triton and NaCl-Triton extracts were separately dialyzed overnight against several changes of buffer D to remove EDTA and NaCl, respectively, and then applied to a column (1.8 × 10 cm) of phosphorylated cellulose previously equilibrated with buffer D. The column was initially washed with 150 ml of buffer D, followed by 400 ml of a 10—500 mM potassium phosphate linear gradient (pH 7.0) containing also 4 mM mercaptoethanol and 0.2% Triton X-100. The flow rate was 60 ml/h and 3.7 ml fractions were collected. The fractions were assayed for casein kinase and histone kinase activities, in the presence and absence of cyclic AMP, by following the procedure described below. All operations were carried out in a cold room at 0—4° C.

Preparation of cytosolic protein kinases. Cytosolic protein kinases S and TS were prepared from human erythrocyte hemolysate as previously described [10].

Assay of protein kinase activity on exogenous proteins (whole casein and histone). Casein (or histone) kinase activity was determined by incubating the enzyme sample (100 μl) at 37°C for 15 min in a medium containing (final volume 0.3 ml) 25 μmol Tris-HCl (pH 7.5), 1.2 μmol $MgCl_2$, 5 nmol [γ-^{32}P]-ATP ($1.5 \cdot 10^5$ cpm/nmol), 0.5 mg casein (or 0.75 mg histone) as substrates. When cyclic AMP was present, its final concentration was 1 μM. The reaction was stopped by addition of 3.6 ml of 10% trichloroacetic acid, 0.4 ml silicotungstic acid solution [11] and 0.1 mg phosvitin (as carrier), followed by centrifugation. The precipitate was washed four times with 5 ml of the above stopping solution. During the last washing the precipitate was kept at 90°C for 20 min before centrifugation. The pellet was finally dissolved in Instagel scintillation liquid and counted in a Packard Tri-Carb model 3375.

Assay of endogenous phosphorylation. The endogenous phosphorylation of membranes (or EDTA extract) was tested by incubating at 37°C the reaction mixture containing in a final volume of 0.3 ml: 300 μg of membrane proteins (or a volume of EDTA extract obtained from the same amount of membranes), 1 nmol of [γ-^{32}P]ATP ($2 \cdot 10^6$ cpm/nmol), 1.2 μmol $MgCl_2$ and 25 μmol Tris-HCl buffer, pH 7.5. The incubation was stopped by the addition of 1% SDS. An aliquot of the solubilized membranes (approx. 40 μg) or of the EDTA extract was then subjected to electrophoresis on SDS-polyacrylamide (5.6%)

gels according to the procedure of Fairbanks et al. [9]. The gels were stained for proteins with Coomassie brilliant blue [9].

Radioautography. The gels were sliced longitudinally, dried at 37°C on a permeable cellophane sheet and exposed to Kodak Medical X-O-Mat X-ray film (Kodak Spa, Milan).

Electrophoresis of ^{32}P-labeled protein hydrolysates. When the ^{32}P-labeled membranes or EDTA-extracted proteins had to be hydrolyzed for Ser-*P*/Thr-*P* analysis, they were precipitated and washed with trichloroacetic acid and silicotungstic acid as described above for the assay of casein and histone kinase activity. The final pellet was hydrolyzed in 2 ml of 6 M HCl for 4 h at 105°C and then dried and subjected to electrophoresis as previously described [12].

[γ-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham, U.K.; phenylmethylsulfonyl fluoride, histone IIA and cyclic AMP were purchased from Sigma Chemical Co., St. Louis; ATP was purchased from Boehringer Mannheim; casein (Hammarsten) was obtained from Merck and purified as previously described [10].

Protein determination. The protein content in the absence of Triton X-100 was determined by using the method of Lowry et al. [13], while in the presence of Triton X-100 the procedure of Wang and Smith [14] was followed.

Results and Discussion

Previous results have shown that in human erythrocytes, membrane-bound protein kinase activity is due to different forms which phosphorylate, in addition to endogenous membrane proteins [1], exogenous proteins such as whole casein and histone, through a cyclic AMP-independent mechanism [10]. These different protein kinases can be solubilized from the membranes by 0.1 mM EDTA (pH 8), but not completely so as indicated by the findings that most of the protein kinase activity can be removed by washing with 0.8 M NaCl the membranes previously extracted with EDTA and that further protein kinase activity is still retained by the 0.8 M NaCl-extracted membranes and solubilized only by Triton X-100.

In contrast, extraction performed twice with EDTA completely removes spectrin from the membranes, as indicated by its absence in the residual extracted membranes (Fig. 1).

Spectrin can be easily separated from protein kinases by submitting the EDTA-extract to Sepharose 6B filtration at high ionic strength (0.5 M NaCl) (Fig. 2a).

It can be seen that spectrin is eluted at a void volume, V_o, as shown by the SDS-polyacrylamide electrophoresis patterns of the eluted fractions indicated by the dashed arrows and shown on the right-hand side of Fig. 2a.

Conversely, protein kinase activity is eluted in a more retarded fraction phosphorylating both histones and whole casein. A similar fraction displaying both casein kinase and histone kinase activity is obtained by Sepharose 6B filtration (Fig. 2b), under the same conditions, of 0.8 M NaCl-extract from membranes previously extracted by EDTA. It must be noted that, while the histone kinase activity of the crude EDTA-extract is cyclic AMP-dependent, histone kinase activity of the crude 0.8 M NaCl extract and that of the Sepha-

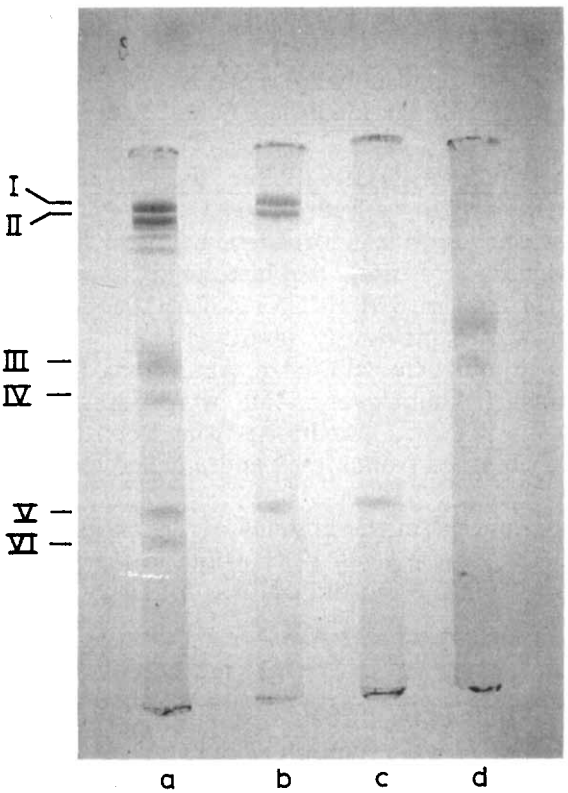


Fig. 1. Electrophoretic patterns on SDS-polyacrylamide gels (5.6%) obtained from: (a) human erythrocyte membranes (about 40 μg protein), (b) 0.1 mM EDTA extract from the membranes, (c) 0.8 M NaCl extract from the membranes previously extracted by 0.1 mM EDTA, (d) residual extracted membranes. The membranes (native or extracted) were solubilized by heating at 100°C for 3 min in 1% SDS, 5—10% sucrose, 10 mM Tris, pH 8, 1 mM EDTA, pH 8, 40 mM dithiothreitol and 0.005% bromophenol blue (tracking dye) and subjected to electrophoresis in 5.6% polyacrylamide gels using a Tris-acetate buffer system, pH 7.4, containing 1% SDS [9]. 1% SDS was added to EDTA and 0.8 M NaCl extracts, before submission to electrophoresis. After electrophoresis, the gels were fixed, stained with Coomassie brilliant blue and destained following the procedure of Fairbanks et al. [9].

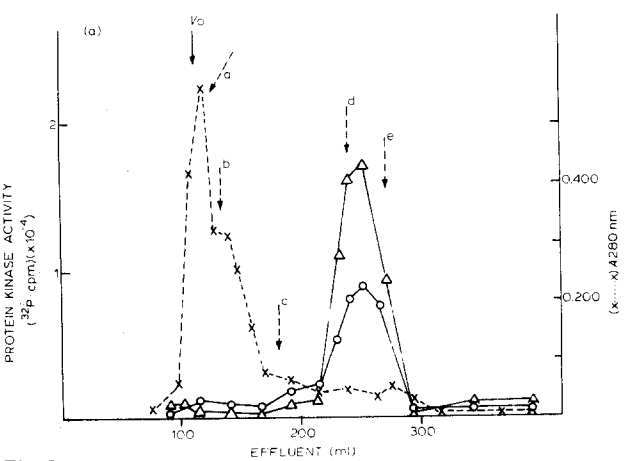
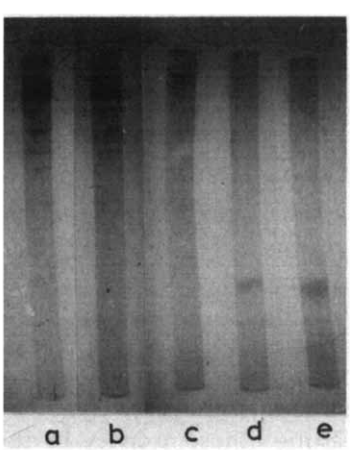


Fig. 2a.



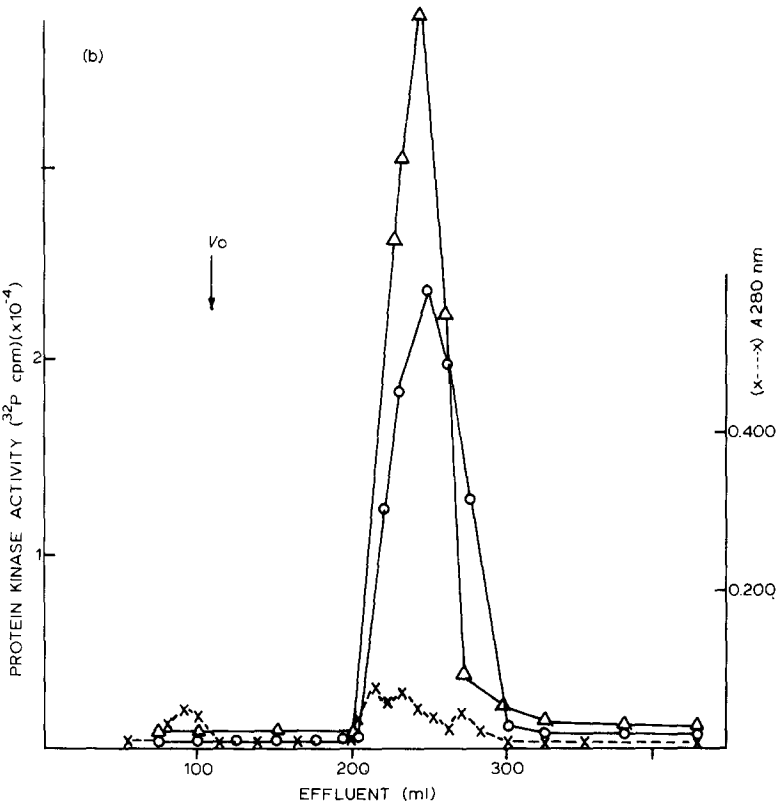


Fig. 2. Elution profile obtained by submission to Sepharose 6B filtration at high ionic strength (0.5 M NaCl): (a) crude 0.1 mM EDTA extract from membranes (about 200 mg), (b) the subsequent 0.8 M NaCl extract from the membranes previously extracted by 0.1 mM EDTA. X-----X, absorbance at 280 nm; ○———○, casein kinase activity; △———△, histone kinase activity. Both protein kinase activities were assayed as described in Methods and Materials and expressed as cpm transferred from [γ-^{32}P]ATP to casein (or histones) under assay conditions. To the right of a are shown the Coomassie blue-stained paterns obtained when 15-μl aliquots of the eluted fractions indicated by the dashed arrows were run on SDS-polyacrylamide gels according to the procedure of Fairbanks et al. [9].

rose fractions obtained from both extracts (Fig. 2a and b) appear to be cyclic AMP-independent, due to the dissociation of the cyclic AMP-binding regulatory subunit from holoenzyme by 0.5 M NaCl [15] of the eluting buffer. On the other hand, high ionic strength is required to avoid the inactivation of casein kinase which occurs during the Sepharose filtration of both extracts at lower ionic strength (0.1 M NaCl) (Fig. 3).

The casein kinase activity of Sepharose fractions obtained by filtration at high ionic strength (0.5 M NaCl) of both EDTA- and 0.8 M NaCl-extracts (Fig. 2a and b), although stable at 0°C for several weeks, undergoes a marked decrease (about 50%) during the overnight dialysis at 0°C against low ionic strength buffer C. On the other hand, such dialysis is required in order to remove NaCl when the two Sepharose fractions are assayed for the ability to phosphorylate spectrin, since NaCl was previously found to inhibit markedly the phosphorylation of membrane proteins by these protein kinase prepara-

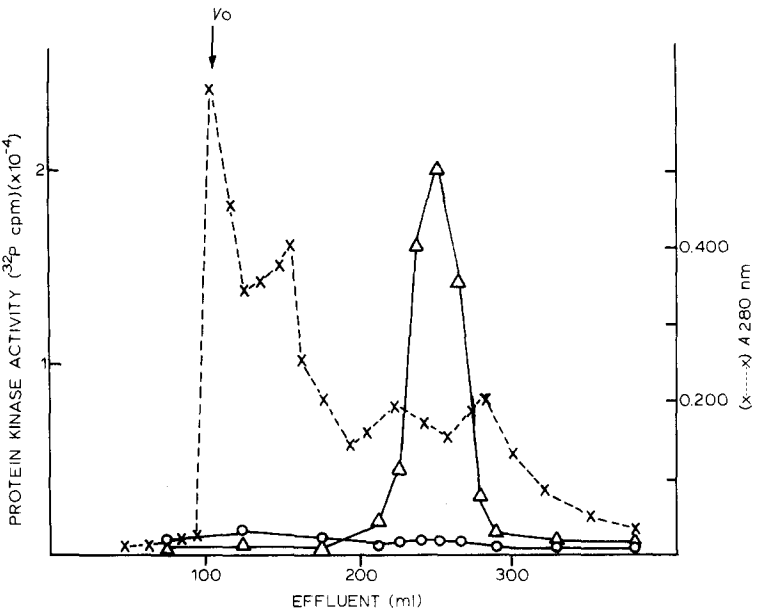


Fig. 3. Elution profile obtained by submitting the crude 0.1 mM EDTA extract from membranes to Sepharose 6B filtration at lower ionic strength (0.1 M NaCl): X------X, absorbance at 280 nm; ○———○, casein kinase activity; △———△, histone kinase activity. Both protein kinase activities were assayed as described in Methods and Materials and expressed as described in the legend to Fig. 2.

tions [1]. Despite such inactivation, both Sepharose fractions, once dialyzed and calibrated on the basis of the same residual casein kinase activity, are still able to phosphorylate, in the presence of [γ-^{32}P]ATP, isolated spectrin to the same extent.

When ^{32}P-labeled spectrin is submitted to SDS-polyacrylamide gel electrophoresis, a pattern showing the typical doublet of spectrin subunits, is obtained (Fig. 4). The radioautography of this electrophoretic pattern clearly shows, in agreement with the finding of other investigators [5,7], that isolated spectrin is phosphorylated by added protein kinases only on the smaller subunit (band II), while the larger subunit (band I) does not appear labeled.

It must be noted that the same result is obtained when spectrin is phosphorylated by the endogenous protein kinases in the isolated membranes or in their crude EDTA extract, incubated in the presence of [γ-^{32}P]ATP (Fig. 5). The latter finding is in contrast with the reports of other investigators [3,4,6] that under such conditions both spectrin subunits are involved in the phosphorylation process.

Most likely, the discrepancy is due to the higher resolution of the two closely sited radioactive bands allowed by the radioautographic technique in comparison with the procedure followed by other investigators, using the slicing of the polyacrylamide gels and subsequent counting of the gel slices.

The results above raised the question as to whether spectrin subunit II was phosphorylated by casein kinase or by histone kinase activity (catalytic subunit), since both these activities were present in our protein kinase preparations

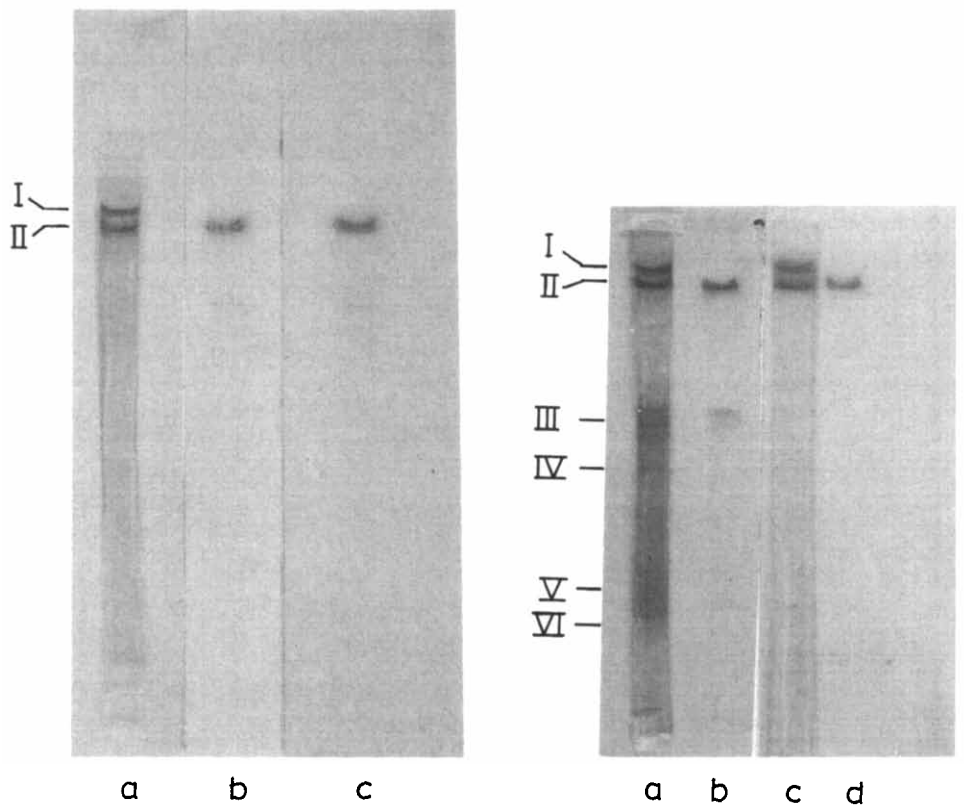


Fig. 4. Phosphorylation of isolated spectrin by added protein kinase solubilized from human erythrocyte membranes. Spectrin was extracted by EDTA and separated by endogenous protein kinases by Sepharose filtration under the conditions described in Methods and Materials. A spectrin sample (100 μg) was incubated for 30 min at 37°C in a medium containing (final volume 0.3 ml): 1 nmol [γ-^{32}P]ATP ($2 \cdot 10^6$ cpm/nmol), 1.2 μmol $MgCl_2$, 25 μmol Tris-HCl buffer, pH 7.5, and an amount of protein kinase preparation which (once dialyzed against buffer C) was able to transfer to the casein about 50 000 cpm in 15 min, under the standard conditions described in Methods and Materials. The incubation was stopped by adding 1% SDS (final concentration) and an aliquot of the mixture (containing 20 μg of spectrin) was subjected to SDS-gel electrophoresis under the conditions described in the legend to Fig. 1. (a) Coomassie brilliant blue-stained gel of isolated spectrin phosphorylated by the protein kinase fraction obtained by Sepharose filtration of EDTA extract as described in Fig. 2a. (b) Autoradiogram of gel a. (c) Autoradiographed gel of spectrin phosphorylated by the protein kinase preparation obtained by Sepharose filtration of 0.8 M NaCl extract as described in Fig. 2b. The corresponding stained gel, clearly showing the doublet of isolate spectrin subunits, was identical to a. Both gels b and c were radioautographed by exposing a film to the gels for 12 h.

Fig. 5. Autoradiograms depicting the endogenous phosphorylation of human erythrocyte membranes and their crude 0.1 mM EDTA extracts. Both the membranes (300 μg protein) and EDTA extracts (obtained from the same about of membranes) were separately incubated in the presence of [γ-^{32}P]ATP under the conditions described in Methods and Materials. After incubation, an aliquot (about 40 μg protein) was solubilized by SDS and electrophoresed under the conditions described by Fairbanks et al. [9]. Electrophoretic patterns a and b were obtained from membranes: (a) dried gel stained with Coomassie blue, (b) autoradiogram of gel a. Electrophoretic patterns c and d were obtained from EDTA extract: (c) Coomassie brilliant blue-stained gel, (d) autoradiographed gel c. Autoradiograms b and d were developed from a film that had been exposed for 3 days to dried gels a and c, respectively.

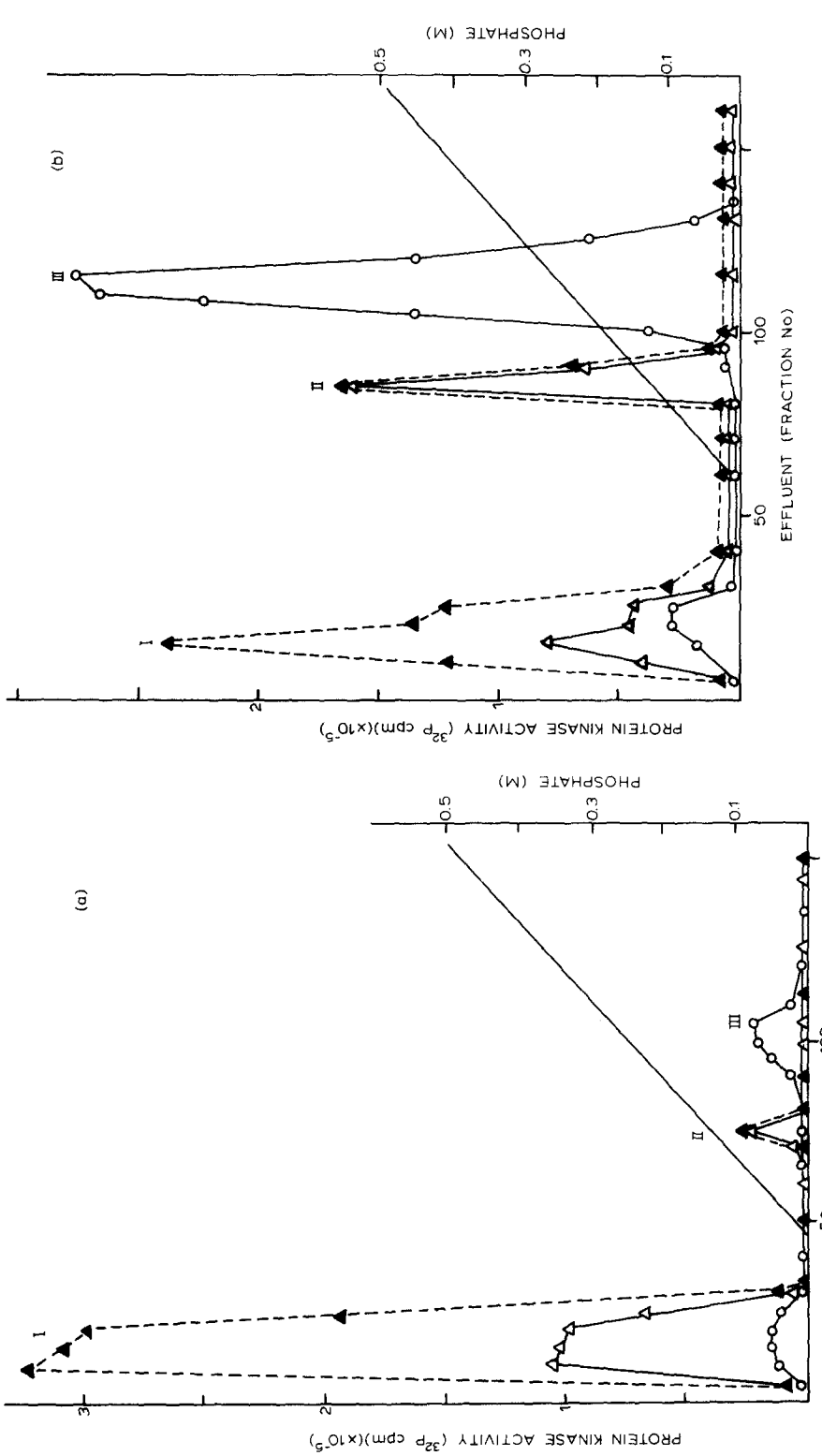


Fig. 6. Chromatographic profile of protein kinase activity on phosphocellulose column displayed by: (a) EDTA-Triton extract, (b) NaCl-Triton extract. The two extracts (90 ml each) were prepared from human erythrocyte membranes (450 mg protein) and, after dialysis against buffer D, submitted to phosphocellulose chromatography under the conditions described in Methods and Materials. The eluted fractions were tested for histone kinase activity in the presence (1 μM) (▲- - - - -▲) and absence (△——△) of cyclic AMP and for casein kinase activity (○——○).

as in that used by Pinder et al. [5] and prepared by following the method of Hosey and Tao [16].

Attempts to separate completely these different protein kinases into stable and more purified preparations have proved unsuccessful, owing to the extreme instability exhibited by these enzymes when the Sepharose fractions are submitted to conventional chromatographic procedures. After many trials, this aim has been achieved by adding 0.2% Triton X-100 to the ionic buffers used for the extraction and subsequent purification by phospho-cellulose chromatography under the conditions described in Methods and Materials. In fact, under these conditions, membrane-bound protein kinase activity is almost completely solubilized by EDTA-Triton and subsequent NaCl-Triton extractions and resolved by phospho-cellulose chromatography into three quite stable fractions which are eluted from the column by different ionic strengths (Fig. 6a and b).

Fraction I, eluted by equilibrating buffer, displays both cyclic AMP-dependent histone kinase activity and casein kinase activity, although the latter is much lower; fraction II, eluted with 0.13 M phosphate, displays only cyclic AMP-independent histone kinase activity; fraction III, eluted with 0.27 M phosphate, contains only casein kinase freed from histone kinase activity.

However, the ratio between the three protein kinase fractions is markedly different in the two subsequent extracts: i.e., in the NaCl-Triton extract fraction III is predominant, while in the EDTA-Triton extract fraction I predominates. When pooled fraction I is treated at 0°C with 0.8 M NaCl for 4 h and

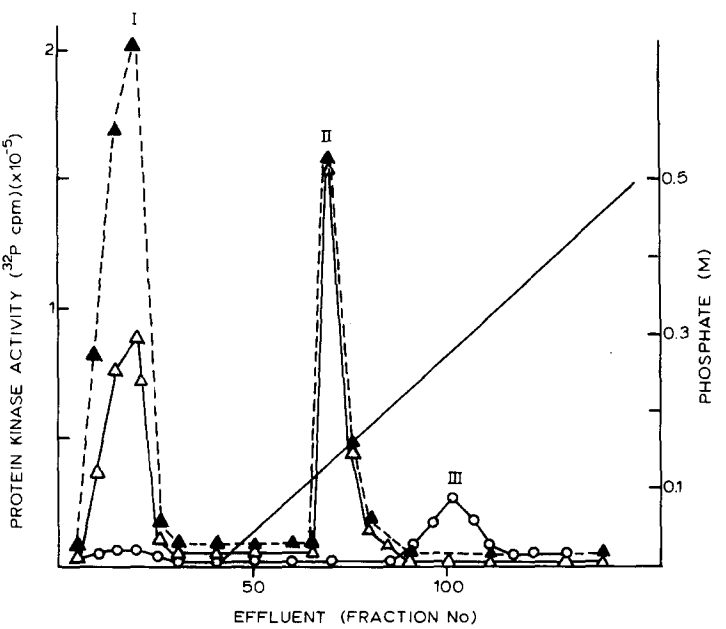


Fig. 7. Elution profile of protein kinase activity obtained when pooled fraction I of Fig. 6b, after preincubation with 0.8 M NaCl and subsequent dialysis to remove NaCl, was resubmitted to phosphocellulose chromatography under the same conditions as in Fig. 6. The eluted fractions were tested for histone kinase activity in the presence (1 μM) (▲- - - - -▲) and absence (△———△) of cyclic AMP and for casein kinase activity (○———○).

then resubmitted (after dialysis to remove NaCl) to phospho-cellulose chromatography, its casein kinase and cyclic AMP-dependent histone kinase activities are partially converted into casein kinase III and cyclic AMP-independent histone kinase II activities, respectively (Fig. 7).

This partial conversion indicates that (1) casein kinase activities eluted in fractions I and III are due to the same enzyme, which in fraction I, is bound to other membrane components to form higher molecular weight aggregates; (2) histone kinase activities eluted in fractions I and II are due to the same cyclic AMP-independent catalytic subunit (eluted in fraction II), which in fraction I, is bound to the regulatory subunit to form the holoenzyme, which is well known to be (unlike the catalytic subunit) cyclic AMP-dependent and is dissociated into the two subunits by the high ionic strength (0.8 M NaCl) of the buffers used for the membrane extraction.

The protein kinase activity exhibited by all three fractions obtained from both Triton-EDTA and Triton-NaCl extracts is quite stable for several months at 0°C.

In conclusion, by adding 0.2% Triton X-100 both to the extracting ionic solutions and to the eluting buffers used for the phospho-cellulose chromatography, it is possible to obtain directly from the crude extracts, without any intermediate step, a stable preparation (fraction III) of casein kinase, completely free of histone kinase activity, together with a stable preparation of histone kinase (catalytic subunit) completely free of casein kinase activity. It must be noted that, under the above conditions, most of the membrane-bound casein kinase activity is recovered into a stable, 200-fold purified preparation, while it is completely lost when the extraction from the membranes and the subsequent phosphocellulose chromatography are carried out with buffers which do not contain Triton X-100. It may be that Triton X-100, by binding hydrophobically to the enzyme molecules, mimics the lipid microenvironment of the enzyme in the structural organization of the native membranes, thus stabilizing the enzyme itself.

Such casein kinase is able to phosphorylate markedly, in the presence of [γ-^{32}P]ATP, isolated spectrin only on its smaller subunit (band II) *. Conversely, histone kinase (fraction II) is completely ineffective, like the histone kinase (lacking in casein kinase activity) obtained, in the absence of Triton X-100, by Sepharose 6B filtration at lower ionic strength of EDTA- and 0.8 M NaCl-extracts (Fig. 3). It is noteworthy that isolated spectrin is markedly phosphorylated only on its smaller subunit (band II) also by the cytosolic casein kinase S prepared as previously described [10], while the cytosolic casein kinase TS is ineffective.

Moreover it is interesting to note that, while whole casein is phosphorylated by both membrane casein kinase and cytosolic casein kinase S only on serine residues [10], isolated spectrin is phosphorylated by both casein kinases on serine and threonine residues, as in intact erythrocytes, by endogenous phosphorylation [17]. The question as to whether or not the isolated spectrin is phosphorylated by the two casein kinases on the same regions of the primary

* This finding is confirmed by a paper [18] which appeared following the completion of this manuscript.

structure which are phosphorylated in intact erythrocytes is under investigation.

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References

1 Michielin, E., Clari, G. and Moret, V. (1978) Int. J. Biochem. 10, 171—175
2 Plut, D.A., Hosey, M.M. and Tao, M. (1978) Eur. J. Biochem. 82, 333—337
3 Avruch, J., Fairbanks, G. and Crapo, L.M. (1976) J. Cell. Physiol. 89, 815—826
4 Greenquist, A.C. and Shohet, S.B. (1974) FEBS Lett. 48, 133—135
5 Pinder, J.C., Bray, D. and Gratzer, W.B. (1977) Nature 270, 752—754
6 Boivin, P. and Galand, C. (1977) FEBS Lett. 79, 91—95
7 Hosey, M.M. and Tao, M. (1977) Biochemistry 16, 4578—4583
8 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
9 Firbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
10 Clari, G., Michielin, E. and Moret, V. (1978) Biochim. Biophys. Acta 539, 420—426
11 Lindberg, O. and Ernster, L. (1956) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. III, p. 8, Interscience, New York
12 Moret, V., Michielin, E., Falezza, G.C. and De Sandre, G. (1977) Clin. Chim. Acta 77, 359—363
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Wang, C.S. and Smith, R.L. (1975) Anal. Biochem. 63, 414—417
15 Boivin, P. and Galand, C. (1978) Biochem. Biophys. Res. Commun. 81, 473—480
16 Hosey, M.M. and Tao, M. (1977) Biochim. Biophys. Acta 482, 348—357
17 Wyatt, J.L., Greenquist, A.C. and Shohet, S.B. (1977) Biochem. Biophys. Res. Commun. 79, 1279—1285
18 Tao, M., Conway, R. and Cheta, S. (1980) J. Biol. Chem. 255, 2563—2568