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PURIFICATION AND CHARACTERIZATION OF AN ADENOSINE CYCLIC 3':5' MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY : A cAMP dependent protein kinase was extracted from human erythrocyte membrane with hydrosoluble fraction and partially purified by ammonium sulfate-precipitation and DEAE-cellulose chromatography. The pH of optimal activity is 6.5 ; the enzyme has an absolute requirement of Mg^{2+} ions at the concentration of 10 mM and is strongly inhibited by Ca^{2+}. It uses ATP as phosphate donor with a Km of 3.7×10^{-6} M. Cyclic AMP stimulates the activity with an apparent Ka of 5×10^{-8} M ; cIMP and cGMP also acts as activators. Enzyme activity is thermolabile and not protected by Mg ATP complex. The enzyme purified from erythrocyte membrane is a type I protein-kinase as proven by DEAE cellulose chromatography and dissociation of the subunits in presence of NaCl 0.5 M and histone.

INTRODUCTION : Endogenous phosphorylation of erythrocyte membrane proteins by membrane protein kinases was first reported by Rubin et al (1) and Guthrow et al (2). Membrane bound protein kinase catalyzes the transfer of ^{32}P from γ (^{32}P) ATP to several endogenous substrates forming stable phosphoproteins. Phosphorylating activity is moderatly stimulated by cAMP and other cyclic nucleotides which bears out the presence of cAMP dependent protein kinase in the erythrocyte membrane. Several endogenous substrates are specifically phosphorylated by this enzyme activity which can also use histone and protamine as substrates.

The present study was an attempt to purify the cAMP dependent protein kinase of human red cell membrane, to study some of its characteristics and compare its properties to those of the other cAMP dependent protein kinases.

MATERIALS AND METHODS

Preparation of ghosts : 200 ml of blood were collected on heparin from normal volunteer donor. Fresh blood was used in order to prevent alteration of membrane enzyme by aging (3). Erythrocytes were washed three times in phosphate buffered isotonic saline (phosphate buffer pH 8, 0.005 M) and buffy coat was carefully removed. Ghosts were prepared following Dodge et al (4) and centrifugated at 18,000 g for 15 minutes.

Purification of cAMP dependent protein kinase : a) 1st step : extraction of membrane proteins.The white pellet of ghosts was transferred

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into a dialysis bag and dialyzed overnight against distillated water + βmercaptoethanol (BME) 1 mM, EDTA 1 mM adjusted to pH 9 with NH_4OH. Ghost suspension was then centrifugated at 100,000 g in a Beckman preparative ultra-centrifuge and the supernatant containing extracted hydrosoluble proteins was collected (fraction I). b) 2e step : ammonium sulfate precipitation. Ammonium sulfate precipitation was performed from the fraction I at 50% saturation for two hours at 4°C. The precipitate was collected by centrifugation at 3,000 g, then 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 in order to remove ammonium sulfate (fraction II). c) 3e step : acidic precipitation. This step was performed in order to eliminate spectrin. Fraction II was acidified by slowly adding acetate buffer 0.5 M pH 5.3. After centrifugation at 3,000 g the precipitate being mainly composed of spectrin was discarded. The supernatant was dialyzed overnight against buffer A and proteins were precipitated by ammonium sulfate at 50% saturation (fraction III). d) 4e step : DEAE cellulose chromatography. DEAE cellulose (DE 52 Whatman) was equilibrated with Tris-Cl buffer 0.005 M pH 7.5 + BME 1 mM + EDTA 1 mM (buffer B) then poured in a little column (Pharmacia) (4 cm x 0.9 cm). Fraction III was dissolved in buffer B and dialyzed against the same buffer ; about 5 mg proteins in 1.5 ml of buffer B were introduced in the column. The column was washed with 25 ml of buffer B. Elution was carried out in a continue concentration gradient of NaCl in buffer B from 0 to 0.4 M with an elution rate of 15 ml/hour. Fractions of 3 ml were collected and assayed for phosphorylating activity (fig. 1). Positive samples were pooled and concentrated to 1.5 ml by filtration on Diaflo PM10 membrane. Purified enzyme was stable for several weeks. Alternatively steps 3 and 4 may be inverted. Acidic preparation was then performed on the pooled positive fractions while always contained a part of the membrane high molecular weight components.

Phosphorylation assays : Phosphorylating activity was assayed with histone as substrate (Histone IIB Sigma) in acetate buffer pH 6.5, 0.05 M following the Guthrow's et al's method slightly modified. Standard assay mixture contained in a total volume of 200 µl in acetate buffer pH 6.5, 0.05 M + EGTA 0.03 M + Magnesium acetate 10 mM : histone 200 µg, $\gamma(^{32}P)$ ATP 0.5 nM (Amersham Center. Specific activity 2-4 Ci/mmol) and when indicated, cAMP 1 nM. For the activity assay of the column elutate 100 µl of enzyme solution were used ; in the other studies 5 µl of purified enzyme were used. Assay mixture was incubated at 30°C for 10 min unless otherwise indicated ; reaction was stopped by adding 4 ml of ice-cold trichloracetic acid (7.5 %) ; the precipitate was dissolved in 0.2 ml 1 N NaOH, reprecipitated in 2 ml trichloracetic acid (5%) and this precipitation-dissolution procedure was repeated a second time. After the final NaOH dissolution the reaction mixture was added to 10 ml of scintillation fluid (Dimilume Packard) and radioactivity was determined by liquid scintillation spectrometry (Intertechnic ABAC SL40). Results of phosphorylation assays were expressed as picomoles of ^{32}P transferred from $\gamma(^{32}P)$ ATP per mg of Histone in ten minutes.

RESULTS

I. Purification procedure (fig. 1)

As previously reported a cAMP dependent protein kinase was extracted from the membrane protein fraction solubilized at low ionic strenght and alkaline pH (5). Protein kinase was precipitated with spectrin by ammonium sulfate at 50% saturation ; however it was not bound to spectrin which was eliminated by acidic precipitation in the present purification procedure ;

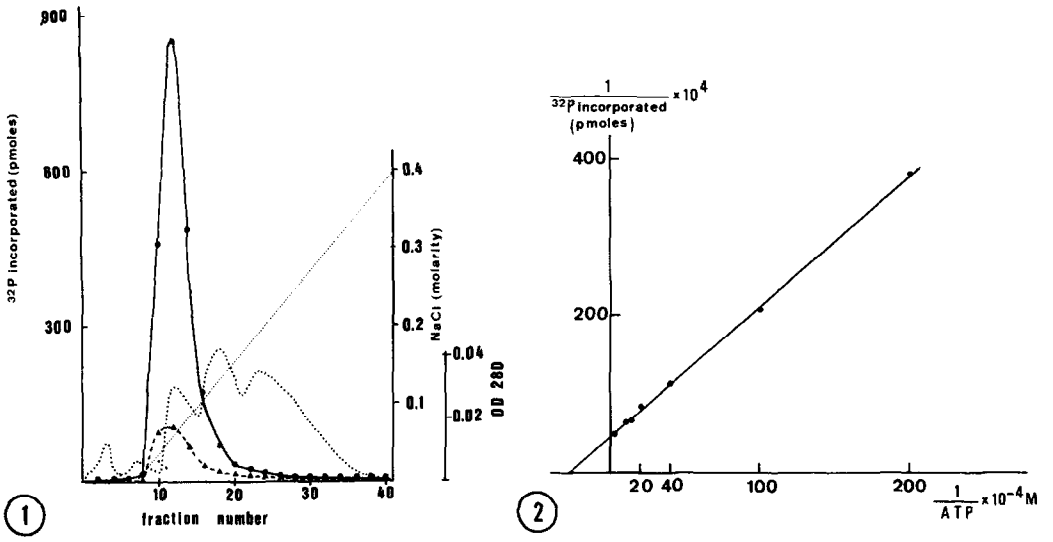


Figure 1

DEAE cellulose chromatography of fraction III (see text) with an elution gradient from 0 to 0.4 M NaCl.
..... optical density of eluate at 280 nm.
▲- - - - - ▲ basal protein kinase activity (without cAMP).
●——● protein kinase activity in presence of cAMP expressed as picomoles of ^{32}P incorporated per mg of histone substrate per 100 µl eluate sample and in 10 minutes incubation.

Figure 2

Lineweaver-Burk plot of the reaction rate with respect to ATP concentrations varying from 0.5×10^{-6} to 50×10^{-6} M. Km = 3.7×10^{-6} M.

indeed acidic precipitate was devoid of phosphorylating activity. cAMP dependent protein kinase was eluted from the DEAE column at 0.05 M NaCl and no other activity peak was detected in the collected fractions. Ratio of the basal activity (without cAMP) to the activity with cAMP added, was varying from 0.06 to 0.1 from one purification to another. Specific activity of purified enzyme was between 600 to 750 nanomoles of ^{32}P transferred, expressed per mg of histone and per mg of enzyme protein and for a 10 minutes incubation-time. Such activity represents an increase by about fifty fold with respect to the specific activity found in hydrosoluble proteins extracted from the membrane. The yield of purification was about 10% and 2/3 of the activity was lost in the acidification step necessary to eliminate spectrin. Enzyme was not purified to homogeneity as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis however the

scarcity of the starting material (fresh human blood) did not allow a complete purification.

II. Kinetic properties

In the standard assay conditions and using 0.8 µg of enzyme protein, the reaction rate with respect to the time was linear for 45 minutes. The effect of pH was studied between pH 5 and pH 10 in a Tris-glycine-maleate buffer 0.06 M. Optimal pH was 6.5.

The enzyme was studied in the standard assay conditions with 7 different ATP concentrations from 0.5 x 10^{-6}M to 50 x 10^{-6} M . Calculated from the Lineweaver-Burk equation, Km ATP was 3.7 x 10^{-6} M (fig. 2). The reaction rate with respect to the cAMP concentrations was studied in the standard assay conditions at 9 different cAMP concentrations between 0.1 x 10^{-8} and 10 x 10^{-8}. The obtained curve was of the sigmoidal type and the cAMP concentration giving half maximal activation was about 5 x 10^{-8}M (fig. 3).

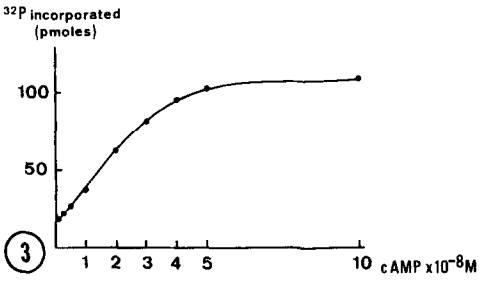
Stimulation by other cyclic nucleotides was studied at final concentrations of 5 x 10^{-6}M for each nucleotide ; activation by cAMP was taken as reference. cIMP stimulated the protein kinase activity to the same extent as cAMP, cGMP activation was 75% of that of cAMP. cUMP, cXMP and cCMP were moderately effective (table 1).

The enzyme showed an absolute requirement of Mg^{2+} ions, with an optimal concentration of 10 mM. It was strongly inhibited by Ca^{2+} ions : activity in presence of 10 mM Mg^{2+} was decreased by 50% with 4 mM Ca^{2+} and reduced to 13% with 10 mM.

For thermostability study, enzyme was incubated at 53°C for 2 to 20 minutes prior to the assay : enzymatic activity was thermolabile and decreased to 10% in 10 minutes. Thermolability was increased when incubation at 53°C was performed in presence of the Mg ATP complexe (Mg^{2+} 8 mM-ATP 0.4 mM).

III. Subunits dissociation

Dissociation of catalytic and regulatory subunits were studied in presence of 0.5 M NaCl by incubating purified enzyme with NaCl at 30°C for 5 to 30 minutes ; assays were performed in the standard conditions with and without cAMP and dissociation was appreciated by the ratio activity without cAMP/activity with cAMP. The dissociation curve with respect to the time was biphasic with a first segment corresponding to the rapid dissociation of about 50% to 60% of the enzyme and a second part responding to a very slow dissociation (fig. 4) which was complete in about two hours.



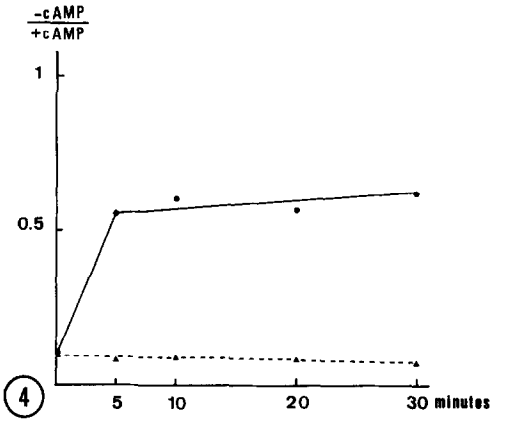


Figure 3

Kinetics of the protein kinase reaction with respect to the cAMP concentrations

Figure 4

Dissociation of catalytic and regulatory subunits by 0.5 M NaCl with respect to the incubation time. Results are expressed as ratio activity without cAMP/activity with cAMP addition.

▲----▲ without NaCl ●——● with NaCl

Table 1 : Stimulation of protein kinase by similar concentrations (5 µM) of nucleotides. Activity was expressed as picomoles of ^{32}P transferred from $\gamma(^{32}P)$ ATP per mg histone/per 10 minutes, using 5 µl of enzyme preparation (0.8 µg of protein) and as per cent of the activity with cAMP addition.

	Protein-kinase activity pM of ^{32}P incorporated	% of activity with cAMP added
Without cyclic nucleotide	56.9	
+ cAMP	420	100
+ cCMP	184	35
+ cGMP	330	75
+ cIMP	445	107
+ cUMP	194	38
+ cXMP	189	36

Histone dissociation performed according to Corbin et al (15) was rapidly complete(fig.5).

DISCUSSION

Some characteristics of the cAMP dependent protein kinase activity of the erythrocyte membrane have been studied using intact ghosts as activity source; however such characteristics are under the influence of the enzyme setting in the membrane and its relationship with the other membrane components. Furthermore activity in intact membrane might be the sum of several isozymic forms of cAMP dependent protein kinase in addition to the cAMP independent activity. The enzyme has been tentatively extracted by the Triton X100 borate method (6) which preferentially solubilize the component III, IV and VI (7), by ghost exposure to high salt concentration (1) and by aquous elution of the spectrin complex (8). However it was not purified further. cAMP dependent protein kinase extracted from human erythrocyte membrane and partially purified possesses the general properties of the cAMP dependent protein kinase bound to the membrane (1, 2, 8, 9). It uses histone as substrate, is dependent of the Mg^{2+} ions, inhibited by Ca^{2+}, stimulated by cAMP, cIMP and cGMP and its optimal pH is 6.5. The Km for ATP was lower than that reported for membrane bound enzyme (2) ; the apparent Ka for cAMP was in keeping with the result of Rubin et al (1) but much lower than that reported by Guthrow et al (2). Mg^{2+} requirement is similar to that reported but the Ca^{2+} concentrations necessary to inhibit purified enzyme are higher than those giving inhibition of the total membrane enzyme (9).

cAMP dependent protein kinase of human erythrocyte membrane is different from the protein kinases extracted from lysates of rabbit reticulocytes (10, 11) and erythrocytes (12). Several of these enzymes are AMP cyclic independent and the properties of the cAMP dependent isozymes are different from those of the human erythrocyte membrane (13, 14) : for instance optimal pH of these enzymes is 8.5 and some are protected by Mg^{2+} ATP against heat inactivation.

cAMP dependent protein kinases exist in mammalian as multiple isozymes, different one from another among species, organs and even the same tissue. Two main types of isozymes have been described and referred to as type I and type II according to their order of elution from DEAE-cellulose with increasing salt concentration and by the ability of high salt concentration or histones to dissociate holoenzyme in its catalytic (C) and regulatory (R) subunits (15).

Elution from DEAE cellulose seems to classify the human erythrocyte cAMP dependent protein kinase as an isozyme of the type I which is eluted

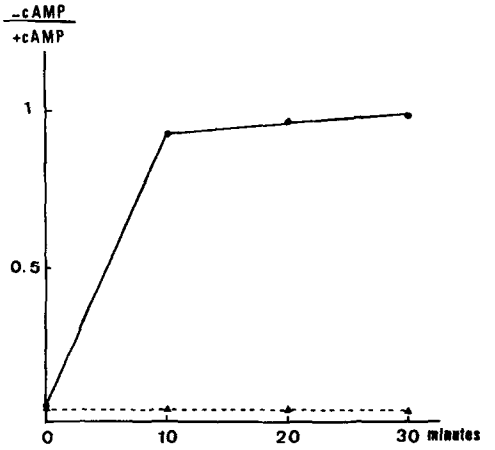


Figure 5

Dissociation of catalytic and regulatory subunits by histone with respect to the incubation time. Results are expressed as ratio : activity without cAMP/activity with cAMP addition.

▲----▲ without histone ●——● with histone

at < 0.1 M NaCl. We have never found any protein kinase activity eluted at 0.2 M NaCl as does type II. This property is shared with the cAMP dependent protein kinase from rat heart, rat liver (15, 16, 17) rabbit skeletal muscle (15, 18), rat testis (15) whereas the type II which is eluted from DEAE at > 0.1 M NaCl is predominant in rat adipose tissue, rat brain, beef heart and pig stomach mucosae (15). Dissociation of the erythrocyte membrane holoenzyme by 0.5 M NaCl and histone is similar to that of the partially purified Type I protein kinase from rat heart. In tissue extracts, differences of isozymic composition have been reported between the cytosol and the membrane fraction (18) : cytosol enzymes are eluted at low salt concentration and membrane enzyme at high concentration ; erythrocyte membrane cAMP dependent protein kinase seems to be similar to the cytosol enzyme and not to the enzyme of membrane tissue fractions. The process of phosphorylation-dephosphorylation seems to be connected to the structural arrangement of membrane components (19) and to the shape and deformability of the red cells (20). cAMP dependent protein kinase is located at the inner surface of the red cell membrane (21, 22) and close to spectrin which is implicated in the physical properties of red cell membrane. The role of the cAMP dependent erythrocyte membrane protein kinase is still unknown. However it is note worthy that in preliminary experiments, purified enzyme was unable to phosphorylate highly spectrin from human erythrocyte membrane.

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