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COMPARTIMENTALIZATION OF SPECTRIN-PHOSPHORYLATING ENZYME IN HUMAN ERYTHROCYTES

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SUMMARY : Functional characteristics of purified casein kinases from cytosol and membrane of normal human red cell were determined in vitro using pure spectrin as substrate. Both enzyme which differed from each other by their salt solubility, molecular weight and affinity for casein had similar affinity for spectrin and same optimum pH ; they used ATP as phosphoryl donor, were similarly stimulated by monovalent cations and Mg^{2+} and inhibited by Ca^{2+} and were insensitive to cyclic nucleotides. Cytosol kinase had a poor substrate and phosphoryl donor specificity ; membrane kinase had a much greater affinity for spectrin than for casein and poorly used GTP as phosphoryl donor : it seems to be the physiologically active spectrin kinase of the red cell.

INTRODUCTION : Endogenous phosphorylation of the spectrin component II occurs in ghosts incubated with $\gamma(^{32}P)$ ATP (1, 2) and in intact red cells incubated with ^{32}Pi (3). Cyclic nucleotide independent casein kinase activity able to phosphorylate spectrin is present in red cell cytosol (4, 5, 6) and membrane (7). It has been suggested that partitioning of this spectrin kinase activity between cytosol and membrane would be under metabolic control and that spectrin phosphorylation might be regulated by translocations of a casein kinase between membrane and cytoplasm (8). Such a hypothesis implies that cytoplasmic casein kinase is identical with the membrane enzyme.

With the aim of determining whether membrane and cytosol activities result from the different locations of a unique enzyme, or depend on two different casein kinases, we have purified casein kinases from the cytoplasm and membrane of human red cells and compared some of their properties using pure spectrin as substrate.

MATERIALS AND METHODS

Blood was collected from patients treated by bleeding for either polycythemia vera or primary hemochromatosis.

Cytosol casein kinase (CCK) was purified to homogeneity as previously reported (9) using ammonium sulfate precipitation, DEAE and phosphocellulose

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chromatography, and affinity chromatography on ATP agarose. Membrane casein kinase (MCK) was extracted from ghosts by 0.5 M NaCl in Tris buffer 5 mM pH 7.5 and then purified by ammonium sulfate precipitation and two successive phosphocellulose chromatographies (10). Pure spectrin was extracted from ghosts by overnight dialysis against a solution of 1 mM EDTA + 1 mM BME alkalized at pH 9 by NH_4OH. It was purified in three steps : ammonium sulfate precipitation at 50% saturation, acidic precipitation at pH 5.3 and gel filtration on a 100 x 1.5 cm column of Ultrogel ACA 34.

Optimal experimental conditions for activity measurement of membrane and cytosol casein kinases have been previously determined (9, 10). Standard assays for membrane casein kinase activity were performed in Tris HCl buffer pH 7.5, 0.05 M + EGTA 0.3 mM + KCl 150 mM + Mg^{2+} acetate 45 mM with casein 2 mg/ml and $\gamma(^{32}P)$ATP 10 µM (final concentration). The mixture used for cytosol kinase assay contained in the same buffer Mg^{2+} acetate 30 mM, KCl 125 mM, with casein 0.4 mg/ml and ATP 10 µM (final concentration). Assay samples of 200 µl were incubated at 30°C for 10 minutes. The reaction was stopped by trichloracetic acid and achieved as previously reported (11). For the assays of both enzymes the spectrin substrate concentration was 1 mg/ml.

Partially dephosphorylated casein was obtained from Sigma, $\gamma(^{32}P)$ ATP (specific activity 2-4 Ci/mmol) and $\gamma(^{32}P)$ GTP (specific activity 4 Ci/mmol) from the Amersham Center ; ATP, GTP and cyclic nucleotides from Boehringer Mannheim, and other chemicals from Merck.

RESULTS

Cytosol and membrane casein kinases differed from each other by their solubility in low ionic strength solution. Cytosol kinase was easily soluble but membrane kinase required the presence of rather high ionic strength ; as previously reported (12) the membrane kinase but not the cytosol enzyme forms aggregates in NaCl or KCl < 0.2 M.

Optimum pH : enzyme activity was assayed in Tris-glycine maleate buffer 0.06 M between pH 5 and pH 9.5. Using spectrin as substrate the optimum pH was pH 7.5 for both enzymes.

Salt stimulation was identical for both enzymes : maximum stimulation was obtained between 0.125 and 0.150 M NaCl or KCl. Concentrations of Mg^{2+}, giving the maximum stimulation were 5 mM for MCK and 10 mM for CCK. Both enzyme activities were inhibited by Ca^{2+}.

Both enzymes use ATP as preferential phosphoryl donor. True Michaelis constants for ATP were determined for both casein kinases using ATP at four different concentrations from 1 to 25 µM, and spectrin at four different concentrations from 0.2 to 1 mg/ml. KmATP was 6.0 µM for membrane kinase and 3.3 µM for cytosol casein kinase.

GTP utilization was determined and compared with ATP utilization in experimental conditions giving optimal activity with spectrin substrate, that is to say, for MCK : in Tris HCl buffer 0.05 M pH 7.5 with spectrin 1 mg/ml,

TABLE 1

Characteristics of pure spectrin phosphorylation by purified membrane and cytosol casein kinases from human erythrocyte and comparison with casein phosphorylation (*ref 10; **ref 9).

	Membrane casein kinase		Cytosol casein kinase	
	Spectrin substrate	Casein substrate*	Spectrin substrate	Casein substrate**
Optimum pH	7.5	7.5 - 8	7.5	6.5
Mg^{2+} stimulation	5 mM	35 - 50 mM	10 mM	25 - 45 mM
Ca^{2+} inhibition	++	++	++	++
Km ATP	6 μM	10.5 μM	3.3 μM	14.7 μM
Km protein substrate	0.59 mg/ml	2 mg/ml	0.55 mg/ml	0.26 mg/ml
GTP utilization	9 %	10 %	73 %	37 %
Salt solubility	in NaCl or KCl $\geq$ 0.2 M*		Easily soluble**	
SDS-PAGE	one subunit 30 000 Daltons MW*		Two subunits 25 000 and 35 000 daltons MW**	

either $\gamma(^{32}P)$ ATP or $\gamma(^{32}P)$ GTP 50 µM, Mg^{2+} acetate 5 mM, KCl 150 mM, EGTA 0.3 mM and for CCK : the same mixture with Mg^{2+} acetate 10 mM.

GTP utilization by membrane kinase was about 10% that of ATP ; it was about 75% for cytosol casein kinase. Both enzymes were quite insensitive to cyclic nucleotides.

Affinity for spectrin was studied at five different ATP concentrations from 1 to 25 µM and four spectrin concentrations from 0.2 to 1 mg/ml. True Michaelis constant calculated from inverse plots was 0.59 mg/ml for MCK and 0.55 mg/ml for CCK.

DISCUSSION

Spectrin endogenous phosphorylation has been studied in ghosts and intact red cells. Avruch and Fairbanks demonstrated that phosphorylation of spectrin component II results from the activity of a membrane salt stimulated cyclic nucleotide independent casein kinase (1).Hosey and Tao (12) extracted casein kinase from rabbit and human cytosol and observed that membrane spectrin may be phosphorylated by cytosol enzyme (6) as well as by membrane enzyme (12). Casein kinase from human red cell cytosol has recently been purified (9) : it resembles the casein kinase II from rabbit reticulocyte cytosol (13) and probably the smaller casein kinase of human erythrocyte cytosol isolated by Michielin et al (14). Human erythrocyte membrane casein kinase has been extracted and concentrated by Hosey and Tao (12) and hightly purified by us (10). However at present, the phosphorylation characteristics of pure spectrin by purified casein kinases from membrane and cytosol have not been determined.

Spectrin phosphorylation by each enzyme differed in some manner from casein phosphorylation : for example optimum pH of cytosol kinase was 6.5 with casein and 7.5 for spectrin substrates ; concentrations of Mg^{2+} giving the maximum stimulation were 5 - 10 mM for both enzymes with spectrin substrate but 45 mM and 30 mM with casein ; Michaelis constant for casein was 2 mg/ml for MCK (10) and 0.26 mg/ml for CCK, very different from the constants observed with spectrin substrate.

Despite of some functional similarities, membrane and cytosol casein kinases from human red cells seem to be two different enzymes, like the membrane and cytosol cAMP dependent protein kinases (15). Varying salt solubility, different molecular weight and subunit composition (9, 10), differing affinities for casein and very different utilization of GTP enables a clear distinction to be made between the two casein kinases. Both enzymes have similar affinity for spectrin when ATP is the phosphoryl donor ; they are therefore able to phospho-

rylate spectrin at a similar extent at least in vitro. However, cytosol casein kinase which has a high affinity for casein is less specific for spectrin than for casein. Furthermore cytosol kinase has a poor specificity for phosphoryldonor as compared with that of membrane kinase which very preferentially uses ATP. Despite the fact that spectrin is in contact to cytosol kinase at the inner surface of the erythrocyte membrane, the membrane casein kinase seems to be really the spectrin kinase.

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