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PURIFICATION AND CHARACTERIZATION OF A CASEIN KINASE FROM HUMAN ERYTHROCYTE CYTOSOL

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SUMMARY : A casein kinase was extracted from human erythrocyte cytosol and purified by ammonium sulfate precipitation, chromatography on DEAE and phosphocellulose, and affinity chromatography on ATP-agarose. This enzyme did not use histone as a substrate ; its activity was not stimulated by cyclic nucleotides. The pH of optimal activity was 6.5. The enzyme had an absolute requirement of Mg^{2+} ions at an optimal concentration of 30 mM ; activity was stimulated by Na^{+} and K^{+} at a maximal concentration of 0.125 M and inhibited by Ca^{2+}. Casein was used as a substrate with a Km of 0.25 mg/ml ; ATP was the preferential phosphoryl donor with a Km of 14.7 µM ; GTP may be used with a lower yield and a Km of 26.3 µM. ADP was a competitive inhibitor of ATP with a Ki of 14 µM. 2-3 DPG was an allosteric inhibitor of ATP with an apparent Ki of 4.6 mM and a Hill coefficient of 3.8. Kinetic data indicate that the reaction follows a coordinated mechanism with ATP as the first substrate and subsequent formation of a ternary complex with the protein. SDS-PAGE of the purified enzyme showed two different peptide chains of molecular weight 35 000 and 25 000.

INTRODUCTION : Cyclic AMP dependent and independent protein kinase activities are present in the membrane and cytosol of human erythrocytes. Red cell membrane contains at least a) a cAMP dependent protein kinase (1-5) type I (6, 7) which is a histone kinase using ATP as phosphoryl donor ; b) a cAMP independent casein kinase activity using ATP or GTP as phosphoryl donor (8, 9). In hemolysates of human red cells, two or three cAMP dependent protein kinase activities have been observed and a cAMP independent casein kinase has been found in lysates of rabbit and human reticulocytes and erythrocytes (10, 11).

Certain human red cell protein kinases have been partially purified previously (6, 12). In this paper, we report the first purification and characterization of the main casein kinase from human erythrocyte cytosol.

MATERIALS AND METHODS

1. Preparation of hemolysate : 300 to 400 ml of blood was collected from patients treated with bleeding for either polycythemia vera or primary hemochromatosis. Erythrocytes were washed three times in phosphate buffered saline (phosphate buffer pH 8., 0.005 M) and the buffy coat was carefully removed. Hemolysis was

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performed at 4°C by adding 1 volume of packed red cells to 15 volumes of phosphate buffer pH 8., 0.005 M. Ghosts were then removed by centrifugation at 18 000 r.p.m. for 15 minutes.

2. Purification of casein kinase

a) First step : ammonium sulfate precipitation

Precipitation of proteins was carried out at 40% saturation $(NH_4)_2SO_4$ for one hour at 4°C. The precipitate was collected by centrifugation at 18 000 r.p.m. for 30 minutes, then washed twice with 40% $(NH_4)_2SO_4$ buffered with phosphate pH 8., 0.005 M + β-mercaptoethanol 1 mM + EDTA 1 mM and kept at 4°C as a suspension in $(NH_4)_2SO_4$. Precipitates obtained from the red cells of 12 patients (1.250 ml of packed red cells) were pooled until the second step of purification was performed.

b) Second step : DE52 chromatography

The pooled precipitates were collected by centrifugation and dissolved in Tris HCl 0.005 M pH 7.2 + β-mercaptoethanol 1 mM + EDTA 1 mM (buffer A) dialyzed for 24 hours against 5 liters of the same buffer. Twenty two milliliters of the protein solution (91 mg per ml) were inserted into a DE52-cellulose column (4.5 cm x 17 cm) previously equilibrated with buffer A. The column was first washed with 120 ml of buffer A, then with 480 ml of NaCl 0.05 M in the same buffer. Elution was carried out in a linear gradient of 1 000 ml NaCl in buffer A from 0.05 to 0.400 M with an elution flux of 80 ml/hour. Fractions of 9.5 ml were collected and assayed for histone and casein kinase activity with an without cAMP. Fractions containing cAMP independent casein kinase activity were pooled and concentrated by ammonium sulfate precipitation at 40% saturation.

c) Third step : phosphocellulose chromatography

Ammonium sulfate precipitate was dissolved in 8 ml of Tris HCl 0.05 M pH 7.5 + β-mercaptoethanol 1 mM + NaCl 0.2 M (buffer B) and dialyzed 20 hours against twice 1 liter of the same buffer. 7.5 ml of the protein solution (61.5 mg per ml) were introduced in a phosphocellulose (P 11 Whatman) column (1.5 x 13 cm) previously equilibrated with buffer B. The column was washed with 170 ml of buffer B and eluted with a linear gradient of 300 ml NaCl from 0.2 to 1 M with an elution rate of 25 ml/hour. Fractions of 5 ml were collected and assayed for casein kinase activity. Active fractions were pooled and concentrated by ammonium sulfate precipitation at 40% saturation. Precipitate was dissolved in 1.5 ml of buffer B, + 10% sucrose, dialyzed against twice 100 ml of the same buffer then divided into 20 microliter fractions which were kept frozen at -70°C until utilization. The enzyme obtained from this step of purification was used for the determination of the kinetic characteristics of the casein kinase.

d) Fourth step : affinity chromatography on ATP-agarose

N^8 (aminohexyl) carbamoyl-methyl adenosine triphosphate agarose was equilibrated with Tris HCl buffer 0.005 M pH 7.5 + EGTA 1 mM + BME 1 mM + magnesium acetate 5 mM (buffer C). 100 µl of protein kinase solution (0.42 mg/ml) were dialyzed overnight against the buffer C, then introduced into a little column (0.5 cm x 1.5 cm) of ATP-agarose gel. Elution was carried out with a linear gradient from 0 to 0.5 M KCl in buffer C with an elution flux of 4 ml/hour. Fractions of 0.25 ml were collected and assayed fro casein kinase activity. Only one elution peak was obtained at 0.25 KCl concentration. About 90 % of the activity (introduced into the column) was collected in the eluate.

3. Enzyme assay

Phosphorylation activity was assayed with either histone or casein as substrate in acetate buffer pH 6.5, 0.05 M according to a slightly modified version of the method of Guthrow et al (2). Standard assay mixture for histone-kinase in a total volume of 200 µl in acetate buffer pH 6.5, 0.05 M + EGTA 0.3 mM + magnesium acetate 10 mM contained histone 200 µg, $\gamma(32^P)$ ATP 0.5 nM and when

indicated cAMP 1 nM. For casein kinase assay the mixture contained magnesium acetate 30 mM, KCl 125 mM ; 10 nM ATP and 400 µg casein were used for 200 µl of the assay mixture. Incubation was carried out at 30°C for 10 minutes ; the reaction was stopped by trichloracetic acid and achieved as previously reported (6) and the radioactivity was determined by liquid scintillation spectrometry (Intertechnic ABAC SL 400). Phosphorylating activity was expressed as picomoles of ^{32}P transferred from $\gamma(^{32}P)$ ATP in ten minutes per 200 µg of histone or 400 µg of casein substrate.

4. SDS-PAGE was performed according to Fairbanks et al (13). For the determinatior of molecular weight the standard mixture (Boehringer) contained : β'RNA polymerase MW 165 000, βRNA polymerase MW 155 000, serum albumine MW 68 000, αRNA polymerase MW 39 000, trypsin inhibitor MW 21 500.

5. Partially dephosphorylated casein, histone IIB and ATP-agarose were obtained from Sigma ; $\gamma(^{32}P)$ ATP (specific activity 2-4 Ci/mmol) from Amersham Center ; DEAE cellulose (DE 52) and phosphocellulose (P 11) from Whatman ; all reagents for polyacrylamide gel electrophoresis from Eastman Kodak ; ATP, GTP and cyclic nucleotides from Boehringer Mannheim and other chemicals from Merck.

RESULTS

I. Purification

When assayed as described above, protein kinase activity of the first ammonium sulfate precipitate was composed of two third cAMP independent casein kinase and one third cAMP dependent and independent histone kinases.

Three peaks of protein kinase were eluted from the DEAE cellulose column (fig. 1) : the first one excluded by washing the column with NaCl 50 mM was composed of a cAMP dependent protein kinase similar to the cAMP dependent protein kinase type I from the erythrocyte membrane ; its activity was lower than 10% of the total protein kinase activity eluted from the column. The second peak was eluted at 0.17 M NaCl : cAMP independent protein kinase

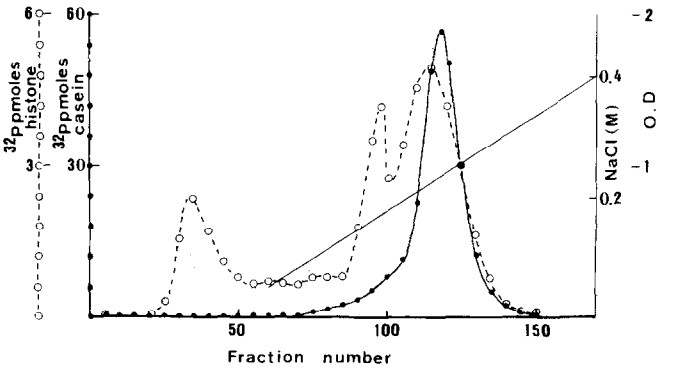

Figure 1 : DEAE cellulose chromatography.
Histone kinase activity was assayed in presence of cAMP. The scale used for the representation of casein kinase activity is one tenth of that used for histone kinase (for details see text).

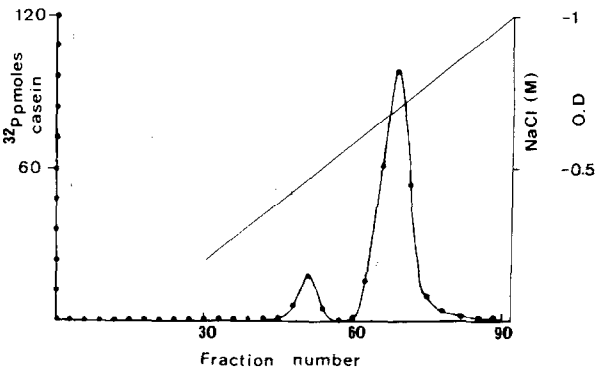

Figure 2 : Phosphocellulose chromatography. (For details see text).

activity was twice as great when casein was used as substrate than when histone was used. More than 80% of the total protein kinase activity was located in the third peak eluted at 0.27 M NaCl. Protein kinase (s) from this peak preferentially used casein as substrate ; the activity with histone was only 6% of that obtained with casein.

Histone kinase activity was eliminated at the next step of purification during the washing of the phosphocellulose column to which it was not fixed (10). Casein kinase was extracted from the phosphocellulose as a major peak at 0.68 M NaCl and a minor component eluted at 0.46 M which accounted for less than 10% of the main component activity (fig. 2). We do not know whether this minor component is an altered form of the main enzyme or a separate casein kinase. Only the fractions responding to the main component have been pooled and subsequently studied.

The enzyme obtained in this way was 55 000 times more concentrated than crude cytosol activity ; the yield was 22 per cent. Its specific activity expressed as reported in "Materials and Methods" was 368 nM/mg protein (table I).

In SDS-PAGE this enzyme preparate showed two main peptide bands of 35 000 and 25 000 molecular weight and some extra bands of higher molecular weight. ATP-agarose chromatography was performed in an attempt to obtain more complete purification. The active fractions eluted from ATP-agarose columns were pooled, concentrated and submitted to SDS-PAGE : they showed two bands similar to those observed in the phosphocellulose column eluate but without any extra bands. These two bands probably correspond to two different peptide chains of the enzyme (fig. 3). When submitted to acrylamide gel electrophoresis without SDS, the enzyme gave rise to agregates which did not penetrate the gel. We were thus unable to use the gradient acrylamide gel method to determine the molecular weight of the enzyme molecule.

TABLE I

Fraction	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	nM ^{32}P	nM ^{32}P/mg	- fold	%
Hemolysate	367500	2453	0.0067		100
$(NH_4)_2SO_4$ 40 %	2000	2237	1.118	167	91.2
DEAE eluate	442.8	1387	3.132	467	56.5
Phophocellulose eluate	1.47	542	368.6	55015	22.1

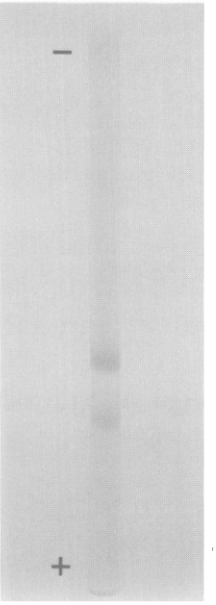

Figure 3 : SDS-PAGE of the enzyme obtained after ATP-agarose affinity chromatography.

II. Properties of the cytosol casein kinase

a) In standard assay conditions and using 0.07 µg of enzyme protein the reaction rate with respect to time was linear for at least 60 minutes. The casein kinase had an absolute requirement for Mg^{2+} with an optimal concentration of 30 millimoles without direct relation to the ATP concentration. It was stimulated by monovalent cations Na^+ and K^+ with an optimal concentra-

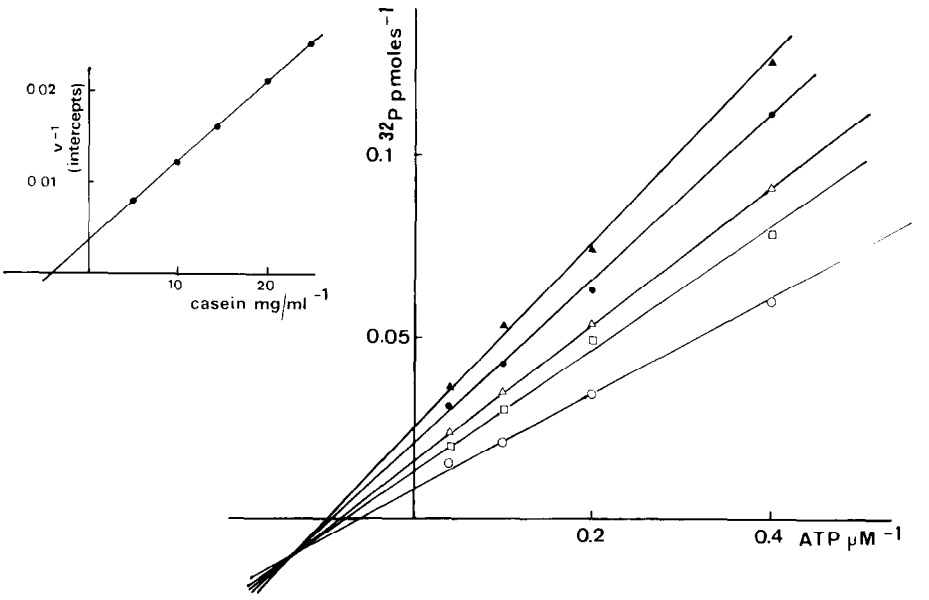

Figure 4 : Lineweaver Burk plot of initial velocity as a function of ATP concentration with casein 0.04 mg/ml (▲——▲), 0.05 mg/ml (●——●), 0.07 mg/ml (△——△), 0.1 mg/ml (□——□) and 0.2 mg/ml (○——○). The insert shows replots of ordinate intercepts versus the reciprocal of the casein concentration.

tion of 0.125 M for K^+ ; concentrations higher than 0.15 M were strongly inhibiting : in presence of KCl 0.3 M activity decreased to 15% of that observed at 0.125 KCl. Calcium ions Ca^{2+} were also inhibiting : activity decreased to 50% for a Ca^{2+} concentration of 12 mM.

b) Optimum pH : Enzyme activity was assayed in Tris-glycine-maleate buffer 0.06 M between pH 5 and 9.5. Optimum pH was 6.5 and the pH curve showed a shoulder at pH 8.5.

c) Protein substrate : The purified protein kinase was a casein kinase ; it did not use histone as substrate : ^{32}P incorporation in histone under experimental conditions similar to those used for casein phosphorylation was nul or insignificant (lower than 1% of incorporation in the casein). Affinity for casein was studied at five casein concentrations from 0.04 to 0.2 mg per ml and at five ATP concentrations from 2.5 to 50 micromoles, in presence of Mg^{2+} (30 mM) and KCl (0.125 M). Michaelis constant calculated from the Lineweaver Burk plots was 0.26 mg per ml (fig. 4).

d) Phosphoryl donor. Effects of nucleotides : The enzyme used ATP as phosphoryl donor. Affinity for ATP was studied at five ATP concentrations from 2.5 to 50 micromoles and five casein concentrations from 0.04 to 0.2 mg/ml. The true Km ATP calculated from the inverse plots was 14.7×10^{-6}M (fig. 5).

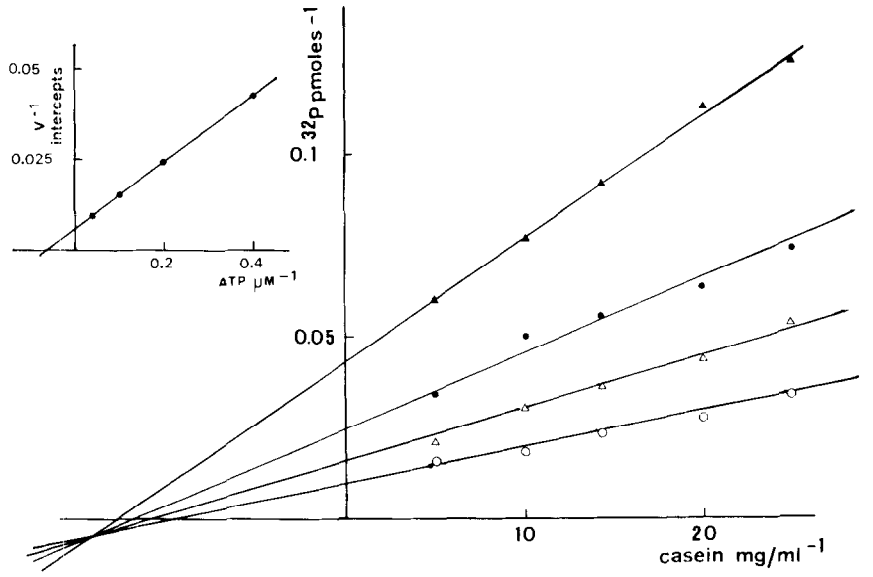

Figure 5 : Lineweaver Burk plot of initial velocity as a function of the casein concentration with ATP 2.5 µM (▲——▲), 5 µM (●——●), 10 µM (△——△) and 50 µM (○——○). The insert shows replots of ordinate intercepts versus the reciprocal of the ATP concentration.

From the above mentioned properties we have determined the optimal standard assay conditions reported in "Materials and Methods". GTP may be used by casein kinase but with a lower yield than ATP : in the standard assay condition utilization of GTP was 37% of that of ATP. Km GTP determined as above was 26.3 x 10^{-6}M. Cyclic nucleotides are without any stimulating effect on the casein kinase activity. Interaction between the substrate ATP and the reaction product ADP was studied by determining the reaction rate in presence of casein 0.2 mg/ml and 2 mg/ml, at five different ADP concentrations from 2 to 20 µM and in presence of four different ATP concentrations from 1 to 7.5 µM. ADP was a competitive inhibitor of ATP with a Ki = 14 µM, whether or not saturating casein concentrations were used. Possible interaction of ADP with casein was studied by measuring the reaction rate with two different ATP concentrations, 2.5 µM and 50 µM, and different concentrations of ADP from 2 to 20 µM and casein from 0.05 to 0.15 mg/ml. As expected, ADP was not an inhibitor when the ATP concentration was saturating and was not a competitive inhibitor of casein when the ATP concentration was not saturating. The above data are summarized in table II.

The kinetic enzyme characteristics with respect to the substrates casein and ATP (ATP-Mg) and ADP inhibition with respect to ATP and casein indicate

TABLE II

varied substrate	fixed substrate	pattern	Ki µM
ATP	casein non saturating	competitive	14
ATP	casein saturating	competitive	14
Casein	ATP non saturating	non competitive	22.2
Casein	ATP saturating	no inhibition	–

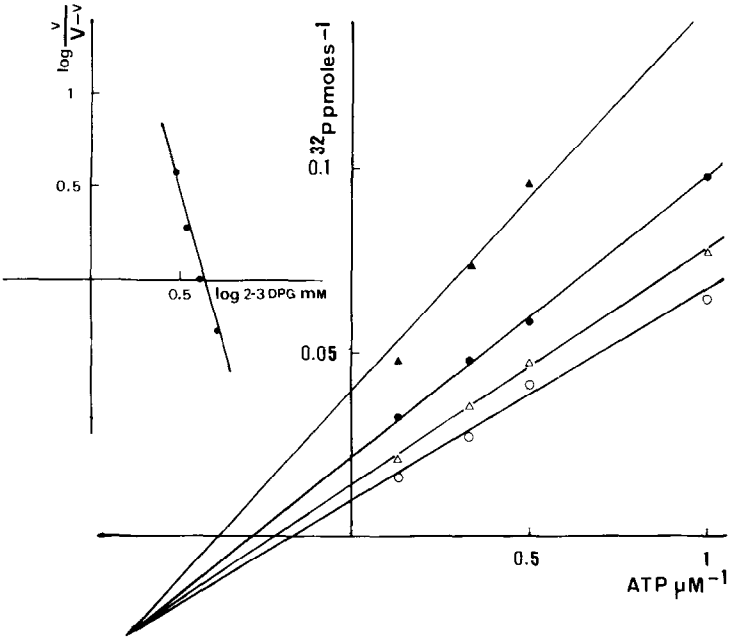

Figure 6 : Product inhibition by 2-3 DPG with ATP as a variable substrate and a non saturating concentration of casein as a fixed substrate. Lineweaver Burk plot of initial velocity as a function of ATP concentration with 2-3 DPG 0 mM (O—O), 3 mM (Δ—Δ), 4 mM (●—●) and 5 mM (▲—▲). The insert shows the Hill plot log (2-3 DPG) = $f\frac{v}{V-v}$: the four slopes are identical.

that the fixation of one substrate is dependent on that of the other and probably follows a coordinated mechanism in which ATP (ATP-Mg) acts as the first substrate, and a ternary complex is subsequently formed with the protein.

e) Inhibition by 2-3 diphosphoglycerate : 2-3 DPG was a potent inhibitor of the casein kinase reaction. In presence of ATP 2.5 µM and casein 2 mg/ml the 50% inhibition was obtained with 2-3 DPG 3.7 mM and total inhibition was observed with a 10 mM concentration. The inhibition curve was a sigmoidal type. Possible interaction between 2-3 DPG and ATP was studied by plotting the

inverse of the reaction rate versus 2-3 DPG concentration from 2.5 to 5 mM at 4 different ATP concentrations from 1 to 7.5 µM. It appears that inhibition by 2-3 DPG with respect to ATP was of allosteric type with a Hill coefficient 3.8 and an inhibition constant Ki : 4.6 mM very near the physiological concentration of 2-3 DPG in the human red cells (fig. 6).

f) Thermal stability : Cytosolic casein kinase was very unstable to heat : activity was decreased to 20% after 10 minutes exposure at 53°C and to 10% after 15 minutes.

DISCUSSION

cAMP independent casein kinase activity is the main protein kinase activity present in human erythrocyte cytosol ; cAMP dependent and independent histone kinase only occurs in less than one third of the different protein kinase activities.

At the present time, purification and characterization of casein kinase(s) from human erythrocyte cytosol has not been reported. Previously published data dealt with enzymes from reticulocytes and erythrocytes of the rabbit. Identification and partial purification of rabbit reticulocyte casein kinase were carried out by Traugh and Traut (10) ; this enzyme shared some properties with human erythrocyte casein kinase, such as the ability to use ATP and GTP as phosphoryl donors and insensitivity to cyclic nucleotides ; however the rabbit enzyme was not purified further and its kinetic properties were not established. Kumar and Tao (11) extracted two casein kinases from rabbit red cell cytosol ; their properties differed from those reported by Traugh and Traut in that these enzymes had an optimum pH at 9 and a relatively low affinity for ATP and GTP.

Casein kinase activity of human erythrocyte cytosol is supported by a major component eluted from the phosphocellulose column at 0.68 M NaCl and a minor one eluted at 0.46 M. Complete purification of the major component was achieved by affinity chromatography on ATP-agarose. Analysis of the purified enzyme on SDS-PAGE showed two peptide chains of molecular weight 35 000 and 25 000 suggesting a minimum molecular weight of 60 000 for the enzyme molecule.

The mechanism of the phosphotransferase reaction catalyzed by erythrocyte casein kinase is poorly documented. Our studies seem to indicate an ordinated (Bi-Bi) mechanism.

Cytosolic substrates of casein kinase have not been identified. Enzyme inhibition by 2-3 DPG at concentrations very close to the physiological one suggests regulation by 2-3 DPG of the phosphorylating activity. As demonstrated

by Hosey and Tao (14) rabbit erythrocyte casein kinase can use some membrane proteins as non-specific substrates in vitro. However this has not been proven to occur in vivo. The biological role of cytosol casein kinase in erythrocytes thus remains to be determined.

Note added in proof : Very recently Hathaway and Traugh (1979, J.Biol.Chem. 254 : 762-768) reported complete purification of casein kinases from rabbit reticulocytes. Properties of human cytosol kinase seem to resemble those of the rabbit CK II. However analysis of purified CK II on SDS-PAGE showed three bands of molecular weight 42 000, 38 000 and 24 000.

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