Human Erythrocyte Pyrimidine Nucleoside Monophosphate Kinase

PARTIAL PURIFICATION AND PROPERTIES OF TWO ALLELIC GENE PRODUCTS

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YAO-SHENG TENG
From the Puget Sound Blood Center, Seattle, Washington 98104

SHI-HAN CHEN AND C. RONALD SCOTT
From the Department of Pediatrics, School of Medicine, University of Washington, Seattle, Washington 98195

Human pyrimidine nucleoside monophosphate kinase is a polymorphic enzyme having two allelic gene products, UMPK 1 and UMPK 2, in several populations. A procedure is described for the partial purification of this enzyme from human red blood cells resulting in a 1500-fold purification of the enzyme for UMPK 1 and 583-fold for UMPK 2.

The purified enzyme preparation catalyzed the phosphorylation of UMP, CMP, and dCMP, and used ATP as the preferred phosphate donor. The heavy metals, mercury, and copper, were found to be strong inhibitors of pyrimidine nucleoside monophosphate kinase activity. EDTA was found to protect the enzyme from inactivation by the heavy metals, and 2-mercaptoethanol stabilized the enzyme during purification.

UMPK 1 and UMPK 2 were found to have similar kinetic properties; however, UMPK 2 had a slower electrophoretic mobility and greater thermolability than UMPK 1.

Pyrimidine nucleoside monophosphate kinase (EC 2.7.4.14) catalyzes a phosphoryl transfer from ATP to CMP, UMP, and dCMP, resulting in the formation of ADP and the respective nucleoside diphosphate.

Genetic polymorphism of pyrimidine nucleoside monophosphate kinase, demonstrable by starch gel electrophoresis of human red cell lysates, was reported by Giblett et al. (1). Initially, UMP was used to demonstrate the zymogram in the gel and an abbreviated name, UMPK, was used to represent the gene product. From family studies, evidence was obtained for three allelic genes (UMPK1, UMPK2, and UMPK3) at an autosomal locus. Of the four electrophoretic phenotypes observed, types 1, 2, and 2-1 were shown to represent homozygosity and heterozygosity for two allelic genes, UMPK1 and UMPK2. UMPK1 was found to have three times the catalytic activity of UMPK2 in human red cells.

Although characterization of pyrimidine nucleoside monophosphate kinase from bacteria (2), Tetrahymena pyriformis (3), calf thymus (4), and rat liver (5) has been reported, there is little information on the corresponding enzyme in man. In this study we undertook partial purification of the enzyme from human red cells and compared some of the biochemical properties of the two allelic gene products.

MATERIALS AND METHODS

Human Blood Cells

Red cells of known phenotype UMPK1 were obtained from outdated donor blood which had been stored at 4° for no more than 3 weeks in citrate/phosphate/dextrose solution at the Puget Sound Blood Center in Seattle. Red cells were washed twice with an equal volume of 0.15 M NaCl, followed each time by removal of the leukocyte-rich top layer. The washed red cells were either stored at −20° in a glycerol freezing solution or were homogenized and used immediately for enzyme purification. The only available unit of UMPK2 blood was collected from a person known to have that phenotype, and the red cells were washed twice with an equal volume of 0.15 M NaCl and preserved in the glycerol freezing solution for several months at −20° before use. During the storage period, there was no loss of enzyme activity.

Chemicals

NADH, UMP, CMP, dUMP, dCMP, GMP, dGMP, ATP, AMP, dAMP, EDTA, phosphoenolpyruvate, lactic dehydrogenase, and DEAE-cellulose were obtained from Sigma Chemical Co. Pyruvate kinase was obtained from Boehringer Ltd., and Sephadex G-75 (particle size, 100 to 270 mesh) from Pharmacia, Uppsala.

Enzyme Assay

The enzyme activity was calculated from the initial oxidation rate of NADH at 37°. The decrease of absorbance at 340 nm was recorded on a
Finally, the solution was centrifuged at 10,000 rpm for 10 min and the phosphate buffer and dialyzed overnight against 1 liter of buffer. The collected precipitate was dissolved in 1 ml of 0.04 M NaCl (from 0 to 0.5 M) in 0.1 M phosphate buffer, pH 6.5, and dialyzed against 1 liter of the buffer for several hours. All the procedures were performed either in a cold room at 4° or in an ice bath. Sodium phosphate buffer containing 1 mm 2-mercaptoethanol and 1 mm EDTA was used for purification throughout the experiments.

Preparation of Hemolysate—Hemolysates were prepared from either the saline (0.9% NaCl solution) washed red cells or from red cells which had been stored in glycerol. The latter red cells were centrifuged and the glycerol solution removed by aspiration. The packed red cells of UMPK 1 or UMPK 2 were then lysed by mixing with an equal volume of 5 mm phosphate buffer, pH 6.5, and 0.5 volume of toluene. After vigorous shaking for 5 to 10 min, the mixture was centrifuged at 5,000 rpm. The hemolysate was collected and again centrifuged at 10,000 rpm for 10 min to obtain a clear hemolysate.

DEAE-cellulose Adsorption—DEAE-cellulose (16 g) was equilibrated with 5 mm phosphate buffer, pH 6.5. The washed DEAE-cellulose was transferred to a beaker containing the hemolysate, stirred for a few minutes, and permitted to stand for 30 to 60 min for enzyme-cellulose binding. Then the mixture was filtered through glass fiber and washed with 500 to 1,000 ml of the buffer to remove most of the hemoglobin. The enzyme was eluted three times with 100 ml of 0.1 M phosphate buffer, pH 6.5, containing 1 mm NaCl. The three eluates were then combined.

Fractionation with Ammonium Sulfate—To 300 ml of the eluate, 83 g of ammonium sulfate was gradually added with constant mixing over a 30-min period. After another 30 min of slow stirring, the mixture was centrifuged at 15,000 rpm for 10 min and the precipitate was discarded. To the supernatant fluid (330 ml), 200 g of ammonium sulfate was gradually added with constant mixing over 60 min. The precipitate was collected by centrifugation and dissolved in 5 ml of 0.04 M phosphate buffer, pH 6.5, and dialyzed against 1 liter of the buffer for several hours.

Sephadex G-75 Column Chromatography—Sephadex G-75 was packed in a column (4 × 30 cm) and equilibrated with 0.04 M phosphate buffer, pH 6.5. The elution rate was adjusted to 15 ml/hour. The dialyzed eluate from ammonium sulfate fractionation was carefully collected to the top of the column with a Pasteur pipette and eluted with 0.04 M phosphate buffer, pH 6.5, and dialyzed against 1 liter of the buffer for 10 min to obtain a clear hemolysate.

Enzyme Characteristics—The reaction mixtures used for determining Michaelis constants of UMP, CMP, and ATP were the same as those used for routine enzyme assay, except the substrate concentrations were varied from 2 to 10 times the Km. A Lineweaver-Burk plot was used to estimate the Km.

For inhibition tests, lead nitrate, zinc chloride, cupric sulfate, and mercuric chloride were added to the enzyme assay mixture to a final concentration of 2.5 × 10⁻⁴ M or 2.5 × 10⁻⁵ M.

Temperature stability of each enzyme preparation was determined between 25 to 50°. Enzyme, 0.08 units of activity, was placed in a final volume of 0.04 ml of 0.04 M phosphate buffer, pH 6.4. All assays were performed in duplicate in capped test tubes (10 × 75 mm). To achieve the same quantity of enzyme activity each tube contained either 8 μg of protein from the purified UMPK 1 or 60 μg of protein from the purified UMPK 2. The reaction mixtures were incubated in a covered and gently shaking water bath (Eberbach Co., Ann Arbor, Mich.) for 30 min at three different temperatures; 40, 45, and 50° (+0.1°). A control sample was maintained at 25° for each temperature determination, and the residual activity of this sample was considered as 100% activity. At the end of the incubation period each reaction mixture was immediately immersed in ice water and maintained at 0° until the residual catalytic activity was measured. All enzymatic assays were recorded within 2 hours of the initiation of the temperature inactivation and all assays were performed in duplicate. Ten microliters of the heated samples were routinely used for the assay.

RESULTS

Fig. 1 shows the starch gel electrophoretic patterns of three red cell specimens having the UMPK phenotypes 1, 2-1, and 2. The UMPK 1 band is intensely stained while the UMPK 2 band is slower moving and more weakly stained.

Table I presents a summary of the pyrimidine nucleoside monophosphate kinase purification steps and the DEAE-cellulose column elution pattern of UMPK 1 is shown in Fig. 2. A 1500-fold purification of UMPK 1 and a 480-fold purification of UMPK 2 from hemolysates were achieved. The lower specific activity of the latter enzyme was due to omission of the last purification step (DEAE-cellulose chromatography) necessitated by the limited availability of blood of this phenotype. The storage of the UMPK 2 red cells in the freezing solution may have had an effect on the enzyme activity during the purification process. Also each enzyme was partially inactivated during the purification process, resulting in a lower than expected yield. After starch gel electrophoresis and staining for pyrimidine nucleoside monophosphate kinase activity, each preparation consisted of a single enzyme band corresponding to the original phenotype.

During the purification process it was especially important to remove adenylyl kinase which is present in high concentration in red cells. Since adenylyl kinase is not bound by DEAE-cellulose, over 96% of this enzyme was removed during purification.
Human Erythrocyte Pyrimidine Nucleoside Monophosphate Kinase

Table I

Summary of purification

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Fig. 2. Elution pattern from a DEAE-cellulose column. Partially purified UMP kinase was placed on top of the column and eluted with increasing concentration of NaCl.

The washing of hemoglobin from the DEAE-cellulose/hemolyte mixture. Approximately two-thirds of the guanylate kinase was adsorbed onto the DEAE-cellulose; however, its separation from UMP kinase was more troublesome. In the final preparation GMP only had 7% as much capacity to accept phosphate from ATP as did UMP at the same concentration. This suggested there was some remaining GMP kinase activity.

Throughout the purification procedure, the ratio of enzyme activities using UMP and CMP as substrates remained constant, indicating the presence of a single pyrimidine nucleoside monophosphate kinase in human red cells. This conclusion was enhanced by the finding of corresponding peaks of enzyme activities when UMP and CMP were used as substrates for assaying the eluates obtained from Sephadex G-75 columns. The same isozyme patterns were also observed when either UMP, CMP, or dCMP were used as substrates following electrophoretic separation in starch gel.

The purified enzyme was kept in 1 to 2 ml of 0.04 M phosphate buffer, pH 6.5. The protein concentration was about 0.2 to 0.5 mg/ml and overnight loss of specific activity at 4°C was about 30 to 50%. The enzyme preparation was studied within 2 days after the final purification step.

Properties of Pyrimidine Nucleoside Monophosphate Kinase

Effects of EDTA and 2-Mercaptoethanol—EDTA was found to protect the enzyme from inactivation; at a concentration of 0.5 mM and 10 mM the enzyme activity was 10-fold and 15-fold greater, respectively, than in solutions that did not contain EDTA. We also tested the effects of varied concentrations of 2-mercaptoethanol, and found that, at a concentration of 10 mM, 2-mercaptoethanol significantly enhanced enzyme activity, reaching a maximum rate at 50 mM. However, at lower concentration (below 5 mM), 2-mercaptoethanol had no apparent effect on enzyme activity. These findings were in agreement with previous work of Maness and Orengo (5) on the purified rat liver enzyme.

Michaelis Constants—The Kₘ measurements of each enzyme preparation for UMP, CMP, and ATP is given in Table II. No significant differences between the allelic gene products were observed.

Specificity for Phosphate Donors and Acceptors—ATP, UTP, and CTP were tested as phosphate donors. There was no measurable activity when ATP was replaced by either UTP or CTP at the same concentration. Among the various nucleoside monophosphates tested as phosphate acceptors (Table III) only CMP and dCMP could successfully substitute for UMP. The small amount of activity observed when AMP, dAMP, GMP, and dGMP were used was probably due to trace contamination by adenylate kinase and guanylate kinase. The fact that CMP was only half as effective as UMP could be partially explained by substrate inhibition. The enzyme reached maximal velocity when the CMP substrate concentration was about 0.1 mM;
higher substrate concentrations were inhibitory to enzyme activity.

Maximum velocity of the enzyme was achieved when UMP was used as substrate. At the saturating concentration of 1 mM, the enzyme was twice as active with UMP as with CMP. However, at the lower concentration of 0.01 mM, the enzyme specific activity was greater using CMP rather than UMP as substrate. Since pyrimidine nucleotide levels in red cells are usually extremely low (7), CMP is probably the natural physiological substrate.

**Effects of Various Metals**—The inhibitory effects of various metals on catalysis by the UMPK 1 and UMPK 2 preparations are shown in Table IV. At $2.5 \times 10^{-4}$ M, the inhibition was greater with mercury and copper than with lead or zinc. The most potent inhibitor was mercury, which almost completely suppressed UMPK 1 activity at a concentration of $2.5 \times 10^{-4}$ M.

**Heat Stability of Enzyme**—The purified enzymes were stable at 25° for 30 min with no loss of catalytic activity. As indicated in Fig. 3, however, UMPK 2 lost 70% and UMPK 1 lost 30% of their initial activity at 40° for 30 min. At 45° UMPK 2 had less than 20% residual activity and UMPK 1 had 40% residual activity. At the highest temperature of 50° each enzyme had only 10% residual activity. The temperature inactivation data indicates that UMPK 2 is less stable than UMPK 1 at temperatures near the physiological range. This *in vitro* difference correlates with our *in vivo* findings that UMPK 2 is more labile than UMPK 1 during red cell aging (8).

**DISCUSSION**

There are at least three nucleoside monophosphate kinases in human red cells. A high activity of adenylate kinase was found in human blood cells by Todd *et al.* (9), and Fildes and Harris (10) described a genetic polymorphism of this enzyme in 1966. Guanylate kinase from human erythrocyte was investigated by Agarwal *et al.* (11) and Monn and Christianson (12) and found by the latter not to be genetically polymorphic. Pyrimidine nucleoside monophosphate kinase, the enzyme described in this paper, was found to be polymorphic in 1974 (1). These kinases are probably important for preventing excessive accumulation of nucleoside monophosphates, which are vulnerable to catabolism by 5'-nucleotidase, and for promoting formation of the energy-rich nucleoside triphosphates.

Our efforts to purify red cell pyrimidine nucleoside monophosphate kinase were first hampered by the very low specific activity of the enzyme in hemolysates and by its instability during purification. Finding that EDTA prevented inactivation of the enzyme partially overcame these difficulties and permitted us to monitor catalytic activity by a pyruvate kinase-lactic dehydrogenase coupled assay procedure throughout the purification process. Inclusion of EDTA and 2-mercaptoethanol in buffer solutions used in the purification helped to stabilize the enzyme.

Previous reports on purification and characterization of pyrimidine nucleoside monophosphate kinase from mammalian tissues were those of Sugino *et al.* (4) and Maness and
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The enzyme preparations were incubated for 30 min at each temperature in 0.05 M PO₄ buffer at pH 6.4. Relative activities are expressed in percent as compared to controls maintained at 25° (100%). ---, UMPK 1; ---, UMPK 2.

Orenzo (5). The enzyme purified from calf thymus (4) and rat liver (5) used dCMP, UMP, and CMP as substrates. The respective $K_m$ values for the calf thymus enzyme were $1.4 \times 10^{-8}$ M for dCMP, $8.5 \times 10^{-8}$ M for CMP, and $1.7 \times 10^{-7}$ M for UMP. The $K_m$ values for the enzyme in rat liver were $2.77 \times 10^{-8}$ M for dCMP, $3 \times 10^{-8}$ M for CMP, and $4 \times 10^{-8}$ M for UMP, respectively (5). The corresponding values of the human enzyme for CMP and UMP were $2.6$ and $5.3 \times 10^{-8}$ M, respectively.

The kinetic properties of the $UMPK^1$ and $UMPK^2$ gene products appear to be very similar, as judged by their catalysis of different substrates at varying concentrations. In mature red cells the catalytic activity of UMPK 1 exceeds that of UMPK 2 about 3-fold (1). We have found, however, that in young red cells, lymphocytes and fibroblasts the activity levels of the two gene products differ very little indicating that UMPK 2 is more labile than UMPK 1 during red cell aging (8). Furthermore, in tests of heat stability of the two enzymes UMPK 2 was more readily inactivated than UMPK 1 with the maximum difference being demonstrable near the physiological range.

There is, as yet, no evidence to indicate how the two UMPK allelic gene products differ in molecular structure. Preliminary observations from Sephadex chromatography would suggest that they have a similar molecular weight and therefore may differ from each other by a single amino acid substitution, a characteristic of a majority of allelic gene products. Such a substitution does not appear to have caused a major change in the substrate or allosteric binding sites but it may have altered tertiary structure sufficiently to account for the marked difference in thermal lability and catabolic rate of the two gene products.

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