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STUDIES OF A METHIONINE-ACTIVATING ENZYME

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Abstract—The properties of a methionine-activating enzyme in human erythrocytes and those of other species are described. Several compounds were examined for their capacity to inhibit the enzyme. Electrophoretic mobility of the enzyme from human and animal tissues was investigated by starch block electrophoresis. Possible clinical applications of this new assay are discussed.

METHIONINE-activating enzyme [(-) -L-S-adenosyl methionine synthetase; methionine S-adenosyltransferase] catalyzes the synthesis of S-adenosyl methionine from the substrates, methionine and adenosine triphosphate.[1] The major methyl donor for numerous transmethylation reactions, S-adenosyl methionine contains a "high energy" bond through which it participates in the transmethylation of many physiologic compounds. These reactions include the *N*- and *O*-methylation of catecholamines and indoleamines,[2–5] *N*-methylation of nicotinamide,[6] methylation of nucleotides,[7] and the synthesis of creatinine,[8] phosphotidyl choline[9] and spermidine.[10] Widely distributed in nature and identified in bacterial,[11] plant[9] and mammalian[13] systems, the methionine-activating enzymes isolated and partially purified from such diverse sources have surprisingly similar properties.[13]

Recently three methyl transferases were discovered in the erythrocytes of man and several other species;[14] all required S-adenosyl methionine as methyl donor.[14] These observations prompted an investigation of the capacity of erythrocytes to synthesize S-adenosyl methionine and led to the identification in erythrocytes of a methionine-activating activity, some of whose properties are described in this report.

MATERIALS AND METHODS

Blood and various tissues were obtained from female Sprague–Dawley rats, female Swiss albino mice, male albino guinea pigs and female albino rabbits. Additional blood samples from male dogs, cats and female squirrel monkeys were used. Blood samples were drawn from various normal human volunteers and from patients with various neurologic, ophthalmologic and psychiatric diseases. Human tissues were obtained at autopsy from an accident victim within 6 hr after death.

Heparinized blood was centrifuged at 16,000 *g*. Plasma was discarded and 0·5 ml of the packed cellular elements was diluted to 5 ml with cold glass-distilled water. Radioactively labeled methionine [^{3}H](methyl)-L-methionine of specific activity approximately 125 μc/μmole) purchased from New England Nuclear was purified according

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to the procedure of Volpe and Laster.[15] All chemicals were obtained from commercial sources and were of the highest purity available. *O*-carbamyl-L-serine, *O*-acetyl-L-serine and *l*-aminocyclopentane carboxylic acid were purchased from Cyclo Chemical Corp.

The assay for the methionine-activating enzyme was performed according to a previously described method[16] with the following slight modifications: The incubation mixture consisted of tris–HCl buffer, pH 6·9 (69·5 μmoles), KCl (490 μmoles), $MgCl_2$ (120 μmoles), adenosine triphosphate (7·2 μmoles) and [^{3}H](methyl)-L-methionine (0·031 μmole). In each assay, 500 μl of blood hemolysate was used. Reduced glutathione and unlabeled S-adenosyl methionine did not enhance activity in blood and were therefore omitted. Reaction blanks consisted either of enzyme heated to 95° for 3 min prior to incubation or of a tube from which one substrate (adenosine triphosphate) was omitted from the reaction mixture.

After incubation at 37° for 1 hr, the reaction was stopped by the addition of 10 ml ice-cold water. The entire mixture was then passed over a 0·9 × 3 cm Dowex 50-X-4 (NH_4^+ equilibrated to pH 7·0) column. The column was washed with 10 ml of glass-distilled water and the product eluted with 10 ml of 3 N NH_4OH. One ml eluate was counted in 10 ml of Bray's solution[17] in a Beckman LS 250 liquid scintillation counter. Results are expressed as millimicromoles of S-adenosyl methionine formed per milliliter of packed red blood cells per hour.

To establish identity of the product, ascending paper chromatography was performed on Whatman No. 1 paper in three solvent systems: butanol–acetic acid–water (24:4:10); isopropanol–ammonia–water (8:1:1); 20% KCl–5% acetone–water. Co-chromatography was performed using authentic [^{14}C](methyl)-L-S-adenosyl methionine.

Electrophoresis was performed in a starch supporting medium prepared in 0·05 M sodium barbital buffer, pH 8·6, as described previously.[18] Aliquots of hemolysates of up to 10 ml containing activity of 8–10 μmoles/ml/hr were applied at the origin. Tissues were prepared for application to the starch block by homogenization in 3 vol. of ice-cold glass-distilled water and centrifuged at 100,000 *g* for 1 hr and 3- to 4-ml aliquots containing 5–10 μmoles of activity/ml were applied at the origin. Assay for methionine-activating enzyme activity was performed after the starch block was cut into half-inch segments and after each half-inch segment was eluted with 1 ml of 0·05 M phosphate buffer, pH 7·9. Aliquots (500 μl) of the eluate were then assayed for enzyme activity with the procedure described above. Blood specimens were prepared for electrophoresis by centrifuging at 16,000 *g* for 20 min. Cellular elements were diluted in 3 vol. of water and frozen and thawed twice to ensure lysis. Erythrocyte ghosts were removed by centrifuging at 16,000 *g* for 20 min; 10 ml of the supernatant was shaken vigorously with 1 ml toluene to remove lipids. The toluene, aspirated before application of the hemolysate to the starch block, was shown by comparison to hemolysates prepared without toluene not to diminish enzyme activity. Electrophoresis was performed at 4° for 18 hr, at 360 V and 80 mA.

RESULTS

Incubation of methionine-^{3}H and adenosine triphosphate with hemolyzed blood as described above resulted in the formation of a radioactive product which was identified

as S-adenosyl methionine. Heating the hemolysate for 3 min at 95° prior to incubation or omission of adenosine triphosphate from the reaction mixture produced no activity, thereby demonstrating the enzymatic nature of the reaction. Activity required the presence of magnesium and potassium and was not enhanced by 10^{-3} M reduced glutathione or 10^{-4} M reduced dithiothreitol, but was enhanced 30 per cent by 10^{-3} M cysteine.

Activity examined in various fractions of heparinized blood was observed to be present predominantly in red blood cells. Little or no activity occurred in serum or platelets. A red blood cell fraction depleted of white blood cells by aspiration of the buffy coat showed no diminution of activity. (However, since S-adenosyl methionine is present in white blood cells,[25] it is probable that some of the enzyme is present in white blood cells.) No activity was present in a resuspension of erythrocyte ghosts. Incubation of erythrocytes diluted in normal saline (to preserve cellular integrity) yielded no activity, suggesting that the enzyme is intracellular.

Identification of enzymatically formed S-adenosyl methionine was accomplished in the three solvent systems indicated above. Co-chromatographs of the incubation mixture with adenosine triphosphate were compared for the presence of a peak migrating with the same R_f as authentic co-chromatographed [^{14}C]S-adenosyl methionine. In KCl–acetone–water, authentic S-adenosyl methionine migrated with an R_f of 0·68; in butanol–acetic acid–water, the R_f was 0·39. In isopropanol–ammonia –water, S-adenosyl methionine remained at the origin. Chromatographs of hemolysate mixtures incubated with adenosine triphosphate contained a peak with the same R_f as authentic product. No peak was observed in chromatographs of enzyme preparations incubated without adenosine triphosphate. (A peak migrating close to the solvent front was present in each system and corresponded to authentic [^{3}H]methionine.)

Maximum enzyme activity occurred at the following concentrations: ATP, $0{\cdot}16 \times 10^{-6}$ M; $MgCl_2$, 10×10^{-6} M; KCl, 50×10^{-6} M; methionine, 25×10^{-5} M. Erythrocyte activity remained linear for at least 90 min. Optimum pH was between 7·5 and 8·5 in 0·05 M Tris–HCl buffer. In 0·05 M phosphate buffer, a sharp peak of activity occurred at pH 8·0. No loss of activity was observed after storage at −20° for 5 days or at −80° for 3 weeks.

TABLE 1. IONIC REQUIREMENTS OF THE METHIONINE-ACTIVATING ENZYME

Substitution for potassium at equimolar concentration	
Ion	Per cent activity (K^+ = 100%)*
Cu^+	100
Li^+	56
Hg^+	42
Fe^{2+}	14
Zn^{2+}	12
Na^+	0
Mn^{2+}	0

* 100% Activity was equal to 0·32 ± 0·01 μmoles/ml/hr of product. On any given sample the assay was reproducible to within 3%. Each assay was run in triplicate.

Specificity of the reaction for the nucleotide adenosine triphosphate was demonstrated by showing that substitution of adenosine diphosphate, adenosine monophosphate, 3′5′-cyclic adenosine monophosphate, guanosine triphosphate, guanosine diphosphate or inosine for ATP yielded no activity.

Ethionine, a competitive inhibitor of methionine in this reaction, decreased the radioactive product by 45 per cent at 20 mM. At equimolar concentration with potassium, the monovalent cation Cu^{+} yielded equal activity, whereas Li^{+}, Na^{+} and Hg^{+} were much less active (Table 1). Replacement of Mg^{2+} by Zn^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}, or Mn^{2+} yielded no activity.

Two categories of compounds were examined for their ability to inhibit the erythrocyte enzyme. The first group consisted of analogs of methionine and S-adenosyl methionine. In several different experimental malignancies, inhibitors of the methionine-activating enzyme have been shown to inhibit the growth of tumors.[19,20]

Inhibitors were preincubated with enzyme at room temperature for 10 min prior to assay. Several previously described inhibitors of the enzyme[21] proved to be potent inhibitors of the erythrocyte enzyme. Most potent inhibition was observed with *O*-acetyl-L-serine, L-methionine methyl ester, *O*-carbamyl-L-serine, *l*-aminocyclopentane carboxylic acid and *N*-acetyl-L-serine as well as the aforementioned ethionine (Table 2).

TABLE 2. INHIBITION OF METHIONINE-ACTIVATING ENZYME BY METHIONINE ANALOGUES AT CONCENTRATION OF 20 mM

Compounds	Per cent inhibition*
L-Methionine methyl ester	85
l-aminocyclopentane carboxylic acid	55
O-carbamyl-L-serine	45
L-Ethionine	45
N-acetyl-L-serine	28
O-acetyl-L-serine	18
N-acetyl-DL-methionine	0

* 100% Activity was equal to 0·32 ± 0·01 μmoles/ml/hr of product. On any given sample the assay was reproducible to within 3%. Each assay was run in triplicate.

The second group of compounds examined were drugs known to affect mood or behavior or both. Several reports over the past decade suggested the existence of an abnormality in methionine metabolism,[22] or more specifically transmethylation[23] in the etiology of schizophrenia. Methionine feeding in the presence of monoamine oxidase inhibition reportedly exacerbates schizophrenic symptoms.[22] At concentrations of 20 mM, the following compounds all failed to inhibit: L-tryptophan, 5-hydroxytryptophan, amitriptyline, tryptamine, dimethyl tryptamine, lysergic acid diethylamide, mescaline, cocaine, normorphine, morphine, norepinephrine, normetanephrine, gamma-aminobutyric acid, dextroamphetamine and reserpine.

In humans, sex or age differences failed to affect enzymatic activity and patients with a variety of ophthalmologic, neurologic and psychiatric illnesses exhibited no different enzymatic activities from those of age- and sex-matched controls (to be reported elsewhere).

TABLE 3. ERYTHROCYTE METHIONINE-ACTIVATING ENZYME ACTIVITY IN VARIOUS SPECIES

Species	Activity (μmoles/ml of RBC/hr)
Human	0·32 ± 0·01 (38)*
Rabbit	0·84 ± 0·03 (3)
Mouse	0·38 ± 0·03 (3)
Guinea pig	0·39 ± 0·04 (3)
Cat	0·41 ± 0·02 (3)
Dog	0·52 ± 0·03 (3)
Squirrel monkey	0·67 ± 0·02 (2)

* Number in parentheses is the number of humans or experimental animals.

Methionine-activating enzyme activity was examined in erythrocytes from 38 humans and several common laboratory animals (Table 3). Highest activities occurred in the rabbit and squirrel monkey. Less activity was present in samples from mouse, rat, dog, cat, guinea pig and humans.

Previous work from this laboratory demonstrated electrophoretic heterogeneity of several methyltransferases[24] both in various species and in different tissues from the same species. The starch block was used as a supporting medium. After electrophoresis on a starch supporting medium, the methionine-activating enzyme exhibited surprising homogeneity in several tissues from various species (Figs. 1, 2).

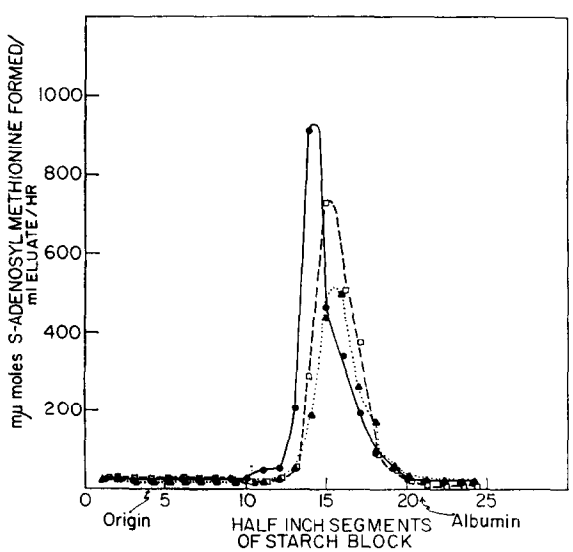

FIG. 1. Starch block electrophoresis of methionine-activating enzyme activity in mouse liver (●), human kidney (□) and human heart (△). Activity is expressed as millimicromoles of S-adenosy methionate formed per milliliter of eluate per hour.

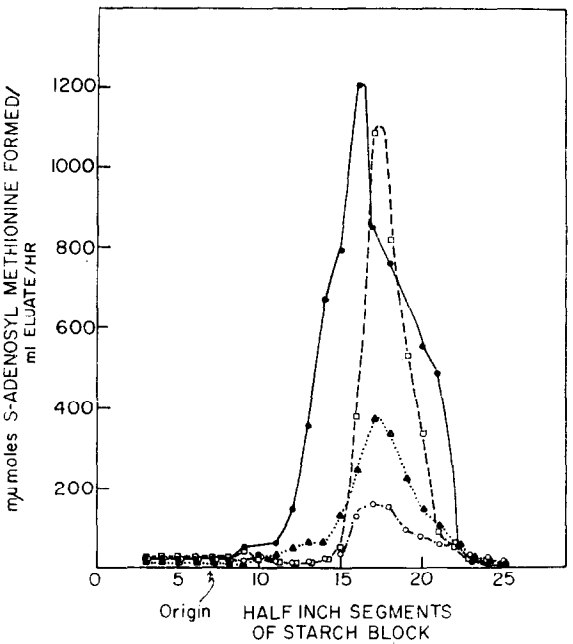

FIG. 2. Starch block electrophoresis of methionine-activating enzyme activity in mouse liver (●), guinea pig liver (△), rabbit liver (□) and human blood (○).

DISCUSSION

A methionine-activating enzyme in human erythrocytes is described. The product of the reaction catalyzed by this enzyme, S-adenosyl methionine, was identified in three chromatographic systems. Substrate specificity, ionic requirements and pH optima are similar in our studies of the erythrocyte enzyme to values reported previously for this enzyme in other tissues and species.[25] The erythrocyte enzyme that we describe, unlike enzymes from other sources,[25] is not stimulated by manganese or zinc as substitutes for magnesium.

The existence of this important enzyme in a readily accessible human tissue permits screening of populations for genetic variants and for alterations of enzyme activity in disease states. Reports of elevated S-adenosyl methionine concentrations in the circulating white blood cells of patients with leukemia[26] and of hypermethionemia and low tissue activity of methionine-activating enzyme in infants with a fatal metabolic abnormality[27] suggest that the enzyme should also be studied in these diseases.

The search for antitumor activity in compounds that inhibit the methionine-activating enzyme may lead to clinical trials of such drugs. The erythrocyte assay permits screening for inhibitors using a human tissue as the source of enzymes. In addition, the erythrocyte assay could permit assessment of inhibition *in vivo* of enzyme activity in patients receiving inhibitors.

The surprising inter- and intra-species homogeneity revealed by starch block electrophoresis contrasts with observations of other enzymes involved in transmethylation.[24] However, more powerful techniques of physicochemical resolution might possibly reveal heterogeneity.

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