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S-Adenosyl-L-methionine Synthetase from Human Erythrocytes: Role in the Regulation of Cellular *S*-Adenosylmethionine Levels†

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ABSTRACT: The properties of human erythrocyte *S*-adenosyl-L-methionine synthetase (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) were studied with respect to the role of *S*-adenosylmethionine in transmethylation reactions. Kinetic values obtained with both a cytosolic and a 350-fold purified preparation of enzyme were compared with measured intracellular concentrations of substrates and products. This analysis revealed that effective regulation of enzyme activity and product concentration can occur through feedback inhibition by *S*-adenosylmethionine (K_i = 2.0–2.9 μM; the endogenous concentration is 3.5 μM). This enzyme can be distinguished from *S*-adenosylmethionine synthetases found in other tissues and appears to be specialized for its role in erythrocyte methyl group metabolism, especially with regard to protein carboxyl methyl-transfer reactions.

S-Adenosyl-L-methionine is the source of methyl groups for a wide variety of biological methyl transfer reactions (Cantoni, 1975). In the red blood cell, this compound has been shown to be the substrate for protein carboxyl methylation reactions (Kim et al., 1980; Freitag & Clarke, 1981) and has been implicated in possible methylation reactions of histamine (Axelrod & Cohn, 1971), catechols (Axelrod & Cohn, 1971; Quiram & Weinshilboum, 1976), and phospholipids (Hirata & Axelrod, 1978). In the human erythrocyte, specific cytoskeletal and membrane proteins are reversibly methylated at D-aspartyl residues (Freitag & Clarke, 1981; McFadden & Clarke, 1982), and it has been proposed that the physiological role of this *S*-adenosylmethionine-dependent reaction is involved in the metabolism of aging proteins (McFadden & Clarke, 1982; Barber & Clarke, 1983).

Red cell *S*-adenosyl-L-methionine is formed in a cytosolic enzymatic reaction from L-methionine and ATP (Cohn et al., 1972; Tallan, 1979). Plasma L-methionine is rapidly equili-

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brated across the red cell membrane (Winter & Christensen, 1964), and ATP is produced by cellular glycolysis. Although the erythrocyte *S*-adenosyl-L-methionine synthetase has not been well characterized, enzymes from *Escherichia coli* (Markham et al., 1980), yeast (Chiang & Cantoni, 1977), and rat liver (β isozyme) (Abe et al., 1982) have been purified, and partial purifications have been reported for other isozymes of rat liver and for tumor tissue enzymes (cf. Liau et al., 1977; Okada et al., 1981).

We were interested in studying the regulatory properties of the erythrocyte enzyme, especially in regard to its role in maintaining a constant intracellular concentration of *S*-adenosylmethionine for protein methylation reactions. We present here a characterization of the human red cell enzyme and demonstrate that its kinetic and structural properties are distinct from those of previously described *S*-adenosylmethionine synthetases in other cells.

Materials and Methods

Preparation of Erythrocyte Cytosol. Fresh, heparinized blood was obtained from volunteer donors. Cells were washed 3 times in 10 volumes of 5 mM sodium phosphate/150 mM NaCl, pH 7.4, and centrifuged at 3400*g* for 10 min at 2 °C. The supernatant and the white cell "buffy coat" were aspirated from the pellet each time. The final erythrocyte pellet was lysed in 4 volumes of deionized water at 0 °C and incubated for 20 min. The membrane fraction was pelleted by centrifugation at 23000*g* for 15 min, and the cytosolic fraction was recovered from the supernatant. The cytosol was dialyzed against 30 mM KCl/40 mM Hepes-KOH,[1] pH 7.4, or 30 mM KCl/40 mM Mes-KOH, pH 5.2, before use.

Assay of S-Adenosyl-L-methionine Synthetase. Enzyme activity was quantitated by measuring the initial rate of formation of *S*-adenosyl-L-[*methyl*-^{3}H]methionine from ATP and L-[*methyl*-^{3}H]methionine by using a procedure modified from those of Mudd et al. (1965), Cohn et al. (1972), and Tallan (1979). Cell extracts were incubated at 37 °C in a total volume of 0.1 mL containing final concentrations of 20 μM L-[*methyl*-^{3}H]methionine (12 600 cpm/nmol; see below for preparation), 10 mM ATP (equine muscle, disodium salt from Sigma), 30 mM $MgCl_2$, 26 mM KCl, and 35 mM Hepes. The final pH was 7.4. Variations to this protocol are noted in the text. After 30–90 min, the reaction was terminated by the addition of 1.0 mL of water at 0 °C, and 1.0 mL of the mixture was applied to a column containing 0.2 mL of acid- and base-washed Dowex AG 50W-X4 resin (Bio-Rad Laboratories) in the NH_4^+ form. Unreacted [*methyl*-^{3}H]methionine was eluted with three 2-mL washes of water; *S*-adenosyl-[*methyl*-^{3}H]methionine was then eluted with 1.0 mL of 3 M NH_4OH into 10 mL of liquid scintillation cocktail (Aquamix, West Chem Products) and counted on a Packard 3255 liquid scintillation counter. Background activity was determined from the radioactivity in samples where water was substituted for enzyme. To minimize this background, it was necessary to purify commercial batches of L-[*methyl*-^{3}H]methionine. Isotope (1.5 μmol, 200 mCi/mmol in 0.3 mL of ethanol/water (7/3); New England Nuclear) was diluted with 13.5 μmol of L-methionine, and the pH was adjusted to 2 with HCl. This material was chromatographed on a 0.9 cm × 30 cm amino acid analysis column (Durrum type DC-6A) which was equilibrated and eluted at 50 °C with sodium citrate buffer (pH 3.25, 0.2 M Na^+) at a flow rate of 70 mL/h. Methionine was eluted at 94–99 min; these fractions were pooled, were made 30 mM in 2-mercaptoethanol, and were stored at −70 °C. The radiochemical purity was determined to be greater than 95% by thin-layer chromatography. The chemical concentration and purity were determined by amino acid analysis.

Chromatography on DEAE-cellulose. Red cell cytosol (30–40 mL, undialyzed) was applied to a column of DEAE-cellulose (1.5 cm × 50 cm, Whatman DE-52) previously equilibrated in 0.1 M potassium phosphate/0.2 mM EDTA, pH 7.0 (Tallan, 1979). The column was washed with 3 resin volumes of this buffer until most of the hemoglobin had eluted. The enzyme was eluted with 3 volumes of 0.2 M potassium phosphate/0.2 mM EDTA, pH 7.0. Active fractions were pooled and concentrated by ultrafiltration (Amicon XM-50). Protein concentrations were determined by first precipitating aliquots of enzyme or serum albumin standards with trichloroacetic acid and then proceeding with a modified Lowry method (Bailey, 1967).

Gel Filtration Chromatography. Red cell cytosol (2–5 mL) was applied to a column of Sephacryl S-300 (0.9 cm × 120 cm) equilibrated at 4 °C in 20 mM potassium phosphate/100 mM KCl, pH 7.4. Fractions (1.5 mL) were assayed for adenosylmethionine synthetase activity and endogenous molecular weight markers. Hemoglobin was assayed by the absorbance at 540 nm. Lactate dehydrogenase, catalase, and carbonic anhydrase were located by their enzymatic activity.

Determination of S-Adenosylmethionine, S-Adenosylhomocysteine, and Methionine by Amino Acid Analysis. Standards of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine (iodide salt) were obtained from Sigma. The procedure of Glazer & Peale (1978) was used to further purify *S*-adenosylmethionine. After lyophilization of the 0.5 M HCl eluant, the material was dissolved in 10 mM HCl and its concentration determined by its ultraviolet spectrum (Schlenk & DePalma, 1957). The concentration of adenosylhomocysteine was confirmed by its ultraviolet absorption (Duerre & Schlenk, 1962).

The concentrations of *S*-adenosylmethionine, *S*-adenosylhomocysteine, and methionine were measured in sulfosalicylic acid extracts of erythrocytes. One volume of packed fresh red cells was diluted with 1 volume of distilled water and then vortexed with 2 volumes of 8% (w/v) 5-sulfosalicylic acid dihydrate (Mallinckrodt analytical reagent). The mixture was allowed to stand at 0 °C for 20 min, and the protein precipitate was then spun out at 2575*g* for 20 min at room temperature. Up to 4 mL of the supernatant was applied directly to the analyzer columns. No correction was made for the volume occupied by the protein pellet in the calculation of the concentration. Analyses were performed on a Beckman Model 120C instrument equipped with high sensitivity cuvettes and recorder so that 10 nmol of aspartic acid gave a full scale response. For *S*-adenosylmethionine and *S*-adenosylhomocysteine, chromatography was done on a 0.9 cm × 10 cm column eluted with 0.40 M sodium citrate buffer at pH 5.55 containing 2.4 M NaCl at 56 °C after the method of Shull et al. (1966). Under these conditions, ammonia eluted at 17 min, *S*-adenosylhomocysteine and arginine eluted at 22 min, and *S*-adenosylmethionine eluted at 32 min. The ninhydrin color constant for *S*-adenosylmethionine was determined to be 0.71 times that of arginine; the minimum detection limit was about 0.2 nmol. *S*-Adenosylhomocysteine was quantitated in separate samples eluted with sodium citrate buffer at pH 4.14 (0.38 M Na^+). Under these conditions, *S*-adenosylhomocysteine elutes at 82–90 min and is well separated from

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DEAE, diethylaminoethyl.

Table I: Characterization of Assay for Erythrocyte *S*-Adenosylmethionine Synthetase[a]

	S-adenosyl[*methyl*-^{3}H]methionine formed [pmol min^{-1} (mg of protein)$^{-1}$] (×10^3)
complete assay mix	130
+10 mM nonisotopically labeled L-methionine	0.2
+25 mM cycloleucine	6.9
−$MgCl_2$, −ATP	1.5

[a] The complete mixture contained 20 mM $MgCl_2$, 20 mM ATP, 25 μM [*methyl*-^{3}H]methionine, 30 mM KCl, 40 mM Hepes-KOH, and dialyzed red cell cytosol (3.3 mg of protein). Incubation was carried out for 60 min.

ammonia, lysine, and histidine (all of which elute between 55 and 65 min) and arginine (which elutes at 125 min). The ninhydrin color constant was 0.86 times that of arginine; the minimum detection limit was about 0.4 nmol. Methionine was quantitated in a standard protocol for neutral and acidic amino acids.

Results

Assay of Erythrocyte S-Adenosyl-L-methionine Synthetase. *S*-Adenosylmethionine synthetase (EC 2.5.1.6) catalyzes the formation of *S*-adenosyl-L-methionine from ATP and L-methionine. Enzyme activity was measured here by the incorporation of radioactivity from L-[*methyl*-^{3}H]methionine into a product which bound to a strong cation-exchange resin and was eluted by 3 M NH_4OH. When enzyme from red cell cytosol was used, little or no incorporation was detected when Mg·ATP was left out of the incubation mixture or when a 400-fold excess of nonisotopically labeled L-methionine was added (Table I). In addition, incubation in the presence of 25 mM cycloleucine, a known competitive inhibitor of *S*-adenosylmethionine synthetases (K_i = 2–6 mM; Sufrin, 1979) reduced incorporation by 95% (Table I). Chromatography of reaction mixtures on the pH 5.55 elution system of the amino acid analyzer described under Materials and Methods revealed that an equivalent amount of radioactivity eluted with a standard of *S*-adenosylmethionine as with NH_4OH when the normal assay procedure was followed. Therefore, we conclude that the material which binds to and is eluted from the Dowex column is authentic *S*-adenosyl[*methyl*-^{3}H]-methionine. The incorporation of radioactivity into this material under the conditions described here was proportional to the time of incubation and the amount of enzyme preparation used (data not shown). Measurements of the enzymatic activity in the erythrocyte membrane fraction indicated that 97.5% of the total cell activity was localized in the cytosol.

Effect of L-Methionine, Mg^{2+}, and ATP Concentration on Enzyme Activity in Red Cell Cytosol. Dialyzed red cell cytosolic extracts were assayed for the initial velocity of *S*-adenosylmethionine synthetase with various concentrations of the substrates Mg^{2+}, ATP, and methionine. The enzyme has a high affinity for methionine; a K_m of 2.0 μM was determined for apparent Michaelis–Menten kinetics (Figure 1).

The catalytic activity of this preparation was found to be dependent on Mg^{2+}. The enzyme is only fully activated at total Mg^{2+} concentrations manyfold higher than the ATP concentration. At near physiological levels of ATP (1 mM), maximal activity is obtained at or above 20 mM $MgCl_2$ (Figure 2). When Mg^{2+} and ATP levels are fixed at 1 mM each, no activation is found with either the addition of 1 mM $CaCl_2$ or 40 mM KCl (data not shown). These latter results suggest

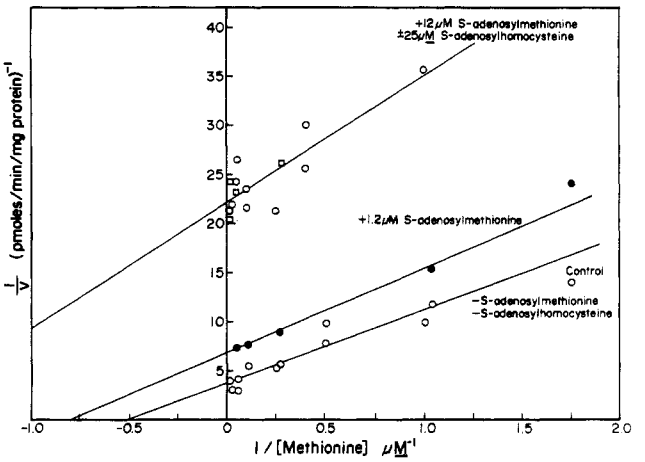

FIGURE 1: Determination of the K_m for methionine of red cell *S*-adenosylmethionine synthetase and the effect of *S*-adenosylhomocysteine and *S*-adenosylmethionine on the enzymatic activity of a cytosolic extract. Aliquots of pH 7.4 dialyzed cytosol (2.7 mg of protein) were assayed in 20 mM Mg·ATP and 0.5–100 μM methionine for 60 min at 37 °C under the following conditions: no added *S*-adenosylmethionine or *S*-adenosylhomocysteine (○); 1.2 μM *S*-adenosylmethionine (●); 12 μM *S*-adenosylmethionine with (□) and without (○) 25 μM *S*-adenosylhomocysteine. A K_m of 2.0 ± 0.7 μM for L-methionine and a K_i of 2.0 ± 0.8 μM for *S*-adenosylmethionine were calculated from a least-squares fit (with 95% confidence limits given) of data for methionine concentrations of 1–100 μM. The latter constant was an average value determined from the *y* intercept of the inhibited lines by using the expression $b = (1/V_{max})(1 + [I]/K_i)$.

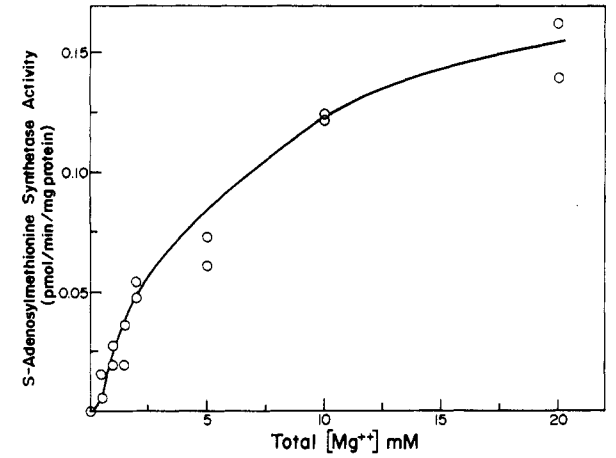

FIGURE 2: Dependence of cytosolic *S*-adenosylmethionine synthetase activity on Mg^{2+}. A dialyzed preparation of cytosol (4.2 mg of protein) was assayed for 30 min at 37 °C with 20 μM L-[*methyl*-^{3}H]-methionine, 1 mM ATP, and various total concentrations of $MgCl_2$ at pH 7.4.

that the activation is not due to a Ca^{2+} impurity in the $MgCl_2$ or a general salt effect. The initial enzymatic velocity was determined for the dialyzed cytosolic enzyme as a function of Mg·ATP concentration, with and without an additional 20 mM concentration of $MgCl_2$ (Figure 3). A K_m for Mg·ATP of approximately 80 μM was calculated from these data when Mg^{2+} was present in excess.

Effect of S-Adenosylmethionine and S-Adenosylhomocysteine on Enzyme Activity in Cytosolic Preparations. Product inhibition by *S*-adenosylmethionine was found to be pronounced when crude cytosol was used as a source of enzyme (Figure 1). This inhibition was uncompetitive with respect to methionine, and an inhibition constant of 2.0 μM was calculated from these data. There was no effect of 25 μM *S*-adenosylhomocysteine, the end product of transmethylation reactions, on either the reaction or the degree of product inhibition by *S*-adenosylmethionine (Figure 1).

Table II: Partial Purification of Erythrocyte *S*-Adenosylmethionine Synthetase

	volume (mL)	activity (pmol min^{-1} mL^{-1})	[protein] (mg/mL)	total activity (pmol/min)	yield (%)	specific activity [pmol min^{-1} (mg of protein)$^{-1}$]	purification (fold)
cytosol	28	5.6	133.5	157	100	0.041	1.0
DEAE pool	42	4.1	0.31	172	109	13.2	322
concd DEAE pool	10	12.3	0.84	123	79	14.6	357

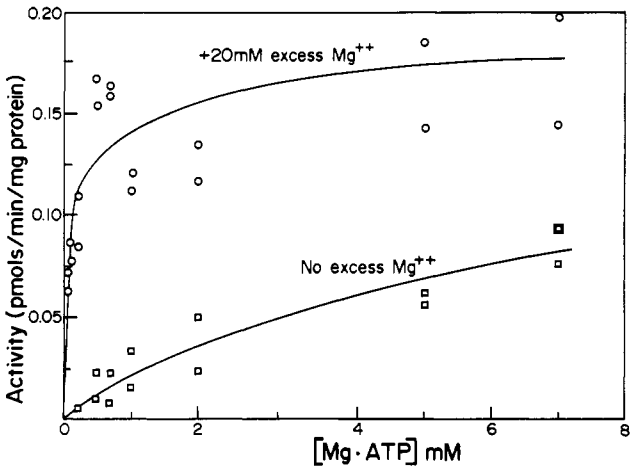

FIGURE 3: Affinity of the cytosolic enzyme for Mg·ATP. A dialyzed preparation of cytosol (4.8 mg of protein) was assayed for 30 min at pH 7.4 with 20 μM methionine and either the indicated equimolar concentrations of ATP and Mg^{2+} or Mg^{2+} in a 20 mM excess over the Mg·ATP concentration. The K_m for Mg·ATP in Mg^{2+} excess was calculated at 81 ± 34 μM from a least-squares fit (with 95% confidence limits) of a double-reciprocal plot.

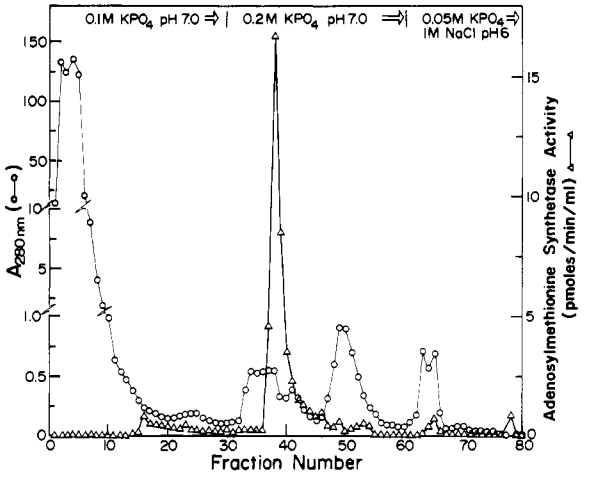

FIGURE 4: Purification of red cell *S*-adenosylmethionine synthetase by DEAE-cellulose chromatography. Erythrocyte cytosol (1480 mg of protein) was fractionated at 2 °C as described under Materials and Methods. The column was eluted with the indicated buffers which also contained 0.2 mM EDTA. Fractions (6.0 mL) were collected and assayed for *S*-adenosylmethionine synthetase activity at pH 7.4 as described. The absorbance at 280 nm of appropriate dilutions was measured for each fraction.

Partial Purification of Red Cell S-Adenosylmethionine Synthetase. The previous kinetic results were obtained with a crude extract of red cells. To reduce the possibility of interfering enzymatic activities, we followed the procedure of Tallan (1979) to fractionate the extract by DEAE-cellulose chromatography (Figure 4). This procedure resulted in an approximate 350-fold purification of the enzyme (Table II). The kinetic properties of the purified enzyme when methionine and *S*-adenosylmethionine are varied are shown in Figure 5. The K_m for methionine is similar to that measured for the crude enzyme (2.2 μM). The degree of product inhibition by *S*-adenosylmethionine was also similar to that measured for

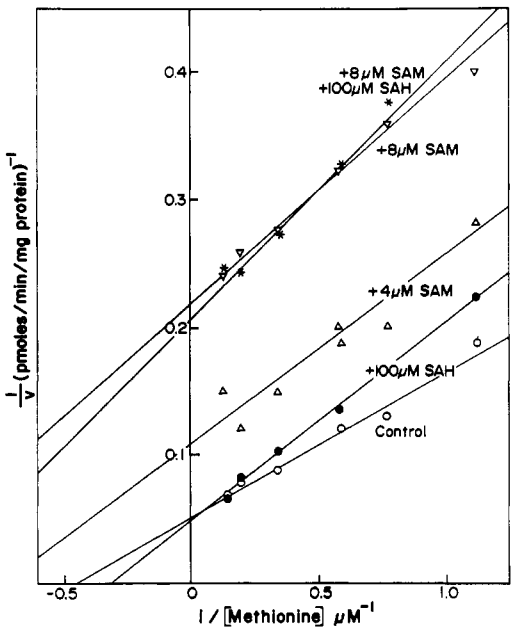

FIGURE 5: Determination of the K_m for methionine and the K_i for *S*-adenosylmethionine for the purified *S*-adenosylmethionine synthetase. Aliquots (21 μg of protein) of the partially purified enzyme were mixed either with no additions (control) or with the indicated concentrations of *S*-adenosylmethionine (SAM) or *S*-adenosylhomocysteine (SAH). These samples were then assayed for *S*-adenosylmethionine synthetase activity in the presence of 40 mM ATP, 40 mM $MgCl_2$, and varying concentrations of L-[*methyl*-^{3}H]-methionine (0.5–10 μM) at pH 7.4. A K_m of methionine of 2.2 ± 0.6 μM and an average K_i for *S*-adenosylmethionine of 2.9 ■ 0.5 μM were calculated from these data as in Figure 1.

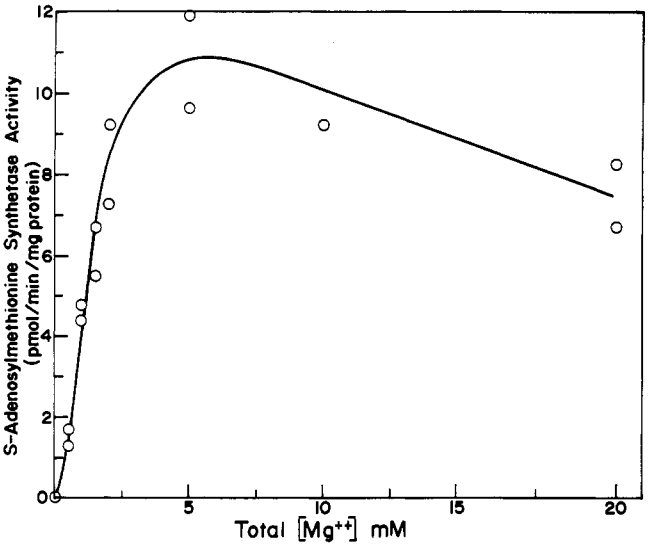

FIGURE 6: Dependence of the activity of partially purified *S*-adenosylmethionine synthetase on Mg^{2+}. Purified enzyme (23 μg of protein) was assayed for 30 min at 37 °C with 20 μM L-[*methyl*-^{3}H]methionine, 1 mM ATP, and various total concentrations of $MgCl_2$ at pH 7.4.

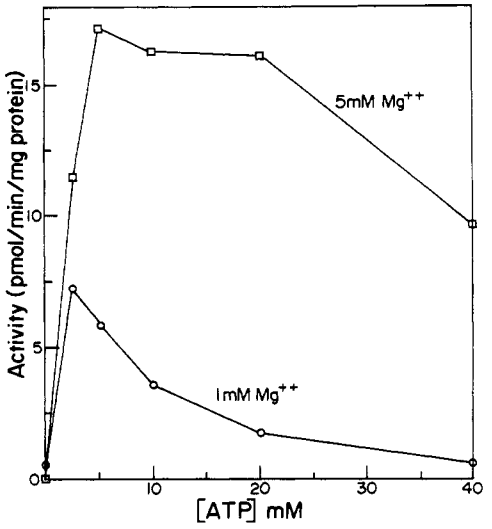

FIGURE 7: Effect of ATP concentration on *S*-adenosylmethionine synthetase activity under conditions of limiting Mg^{2+} concentration. Purified enzyme (36 μg of protein) was assayed for 30 min at 37 °C at pH 7.4 with 20 μM L-[*methyl*-^{3}H]methionine, either 1 or 5 mM total $MgCl_2$, and various concentrations of ATP.

the cytosolic enzyme; an average K_i of 2.9 μM was determined. Again, there was no effect of 100 μM *S*-adenosylhomocysteine on the activity in the presence of *S*-adenosylmethionine. However, in the absence of *S*-adenosylmethionine, apparent competitive inhibition was observed with a K_i of about 200 μM for *S*-adenosylhomocysteine.

The partially purified enzyme is also dependent upon Mg^{2+}. When the ATP concentration is fixed at 1 mM, maximal activity is obtained at 5 mM total Mg^{2+} (Figure 6). To determine whether this might represent a requirement for free Mg^{2+}, the experiment shown in Figure 7 was performed. Here,

Table III: Comparison of Erythrocyte *S*-Adenosylmethionine Synthetase Activity at pH 5.2 and pH 7.4 in the Presence and Absence of *S*-Adenosylmethionine[a]

pH	specific activity of cytosol [pmol min^{-1} (mg of protein)$^{-1}$] control	+125 μM adenosylmethionine	product inhibition (%)
5.2	61.0	46.7	24.4
7.4	137.8	6.9	95.0

[a] Assays were performed as described under Materials and Methods with cytosol dialyzed against 30 mM KCl/40 mM Mes (pH 5.2, 2.5 mg of protein) or 40 mM Hepes (pH 7.4, 2.9 mg of protein). The assay incubation mixtures were also buffered with Mes or Hepes, respectively.

the Mg^{2+} concentration was limiting, and ATP was in excess. The inhibition observed at high ATP and low Mg^{2+} concentrations, where the Mg·ATP complex would be favored at the expense of free Mg^{2+} and the Mg_2·ATP complex (Kohlbrenner & Cross, 1979; Storer & Cornish-Bowden, 1976), indicates that uncomplexed Mg^{2+} and/or the dimagnesium complex is necessary for full activity.

When the enzyme is assayed with varying levels of Mg·ATP, sigmoidal kinetics with an $S_{0.5}$ of 1.1 mM are obtained (Figure 8). In the presence of 2 mM excess $MgCl_2$ (over the concentration of the Mg·ATP complex), hyperbolic Michaelis–Menten kinetics are observed with a K_m of about 450 μM. Intermediate activities are seen when experiments with an excess $MgCl_2$ concentration of 20 mM are performed.

Activity of the Red Cell Enzyme at pH 5.2. In this study, all experiments were performed at the physiological pH of 7.4. In a previous study, Tallan (1979) indicated that the pH optimum of the human erythrocyte enzyme is 5.2 and that the

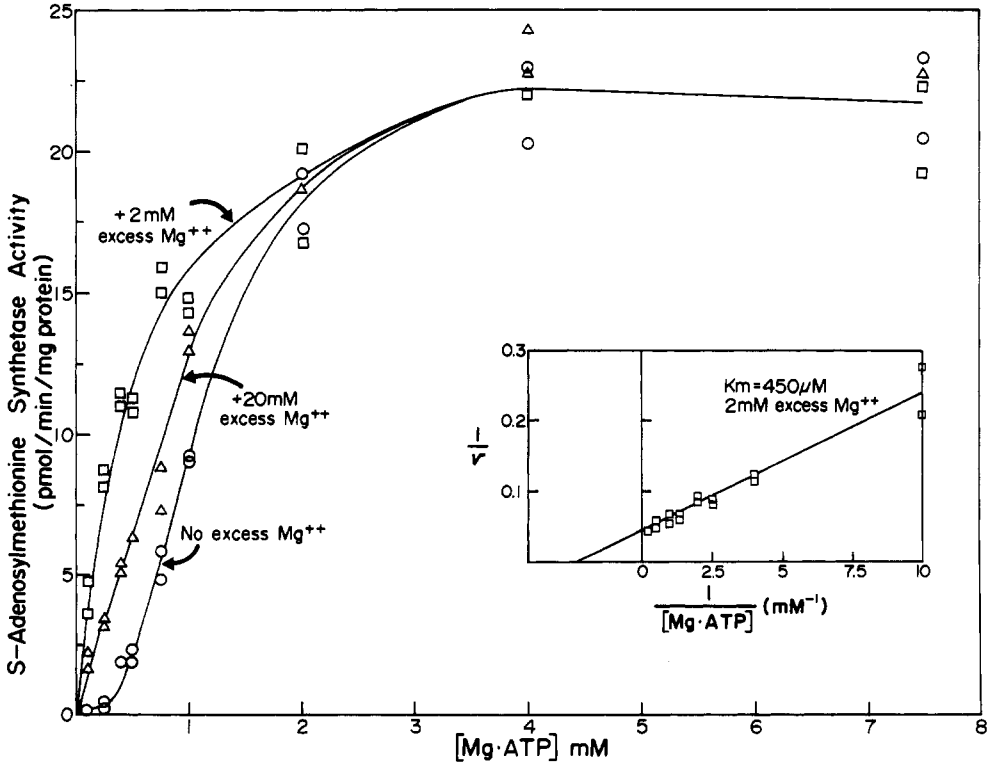

FIGURE 8: Activity of purified *S*-adenosylmethionine synthetase as a function of ATP and Mg^{2+} concentration. Initial velocities were measured at 37 °C, pH 7.4, with 20 μM L-[*methyl*-^{3}H]methionine. Equimolar mixtures of ATP and Mg^{2+} were used for the curve labeled "no excess Mg^{2+}" (○). For the curves labeled "20 mM excess Mg^{2+}" (△) and "2 mM excess Mg^{2+}" (□), the indicated additional amount of $MgCl_2$ was added to the assay mixture. The inset shows a Lineweaver–Burk analysis of the kinetic data from the 2 mM excess Mg^{2+} data. A K_m value of 453 ± 132 μM was calculated from these data as in Figure 1.

Table IV: Physiological Levels of Substrates and Products of *S*-Adenosylmethionine Synthetase in Human Red Blood Cells

	μmol/L packed fresh erythrocytes
methionine	12[a]
	12 ± 7[b]
	24[c]
S-adenosylmethionine	3.5 ± 0.5 (SD) (n = 5)[a]
	3.45 ± 0.5[d]
ATP	1100[e]
Mg^{2+}_{free}	500[e]
S-adenosylhomocysteine	1.3 ± 0.5 (SD) (n = 3)[a]

[a] Determined as described under Methods and Materials. [b] Hagenfeldt & Arvidsson (1980). [c] Leighton et al. (1979). [d] Baldessarini et al. (1978). [e] Jacobasch et al. (1974).

activity at pH 7.4 is only 20% of the maximal activity. However, we have measured the activity of the red cell enzyme at pH 5.2 and find that it is actually less than that measured at pH 7.4 (Table III). The reason for this discrepancy is not clear at present. There is also a significant change in the product inhibition by *S*-adenosylmethionine. A concentration of 125 μM *S*-adenosylmethionine reduces the enzyme activity by 95% at pH 7.4, but only by 24% at pH 5.2. Thus, the regulatory properties of this enzyme appear to be pH dependent.

Determination of the Molecular Size of S-Adenosylmethionine Synthetase by Gel Filtration Chromatography. Figure 9 shows the elution of red cell cytosol fractionated on Sephacryl S-300 resin. A major peak of activity at a molecular weight of approximately 74 000 was obtained, although this assignment is dependent upon the enzyme having the same globular shape as that of the marker proteins. A small peak of activity was detected at an approximate molecular weight of 320 000 in this experiment. In other experiments, there was a small but variable amount of activity between this position and the major peak, which may indicate that the enzyme can exist in different aggregation states.

Physiological Levels of Substrates and Products of S-Adenosylmethionine Synthetase in Human Red Blood Cells. To determine if the kinetic properties of the synthetase measured here, especially the product inhibition observed with *S*-adenosylmethionine (K_i = 2.0–2.9 μM), might be relevant to the activity of the enzyme in intact cells, we measured methionine, *S*-adenosylmethionine, and *S*-adenosylhomocysteine levels in fresh red blood cells (Table IV). The intracellular concentration of methionine determined here (12 μM) is similar to concentrations previously measured (Hagenfeldt & Arvidsson, 1980; Leighton et al., 1979). The value of the intracellular concentration of *S*-adenosylmethionine determined here by amino acid analysis (3.5 μM) is similar to that measured by an enzymatic isotope dilution technique (Baldessarini et al., 1978) but is approximately 10-fold lower than the value reported by Kim et al. (1980). The latter value was obtained by measuring the UV absorbance of a step-elution fraction from a Dowex 50 ion-exchange chromatography column, and this fraction may contain additional UV-absorbing species.

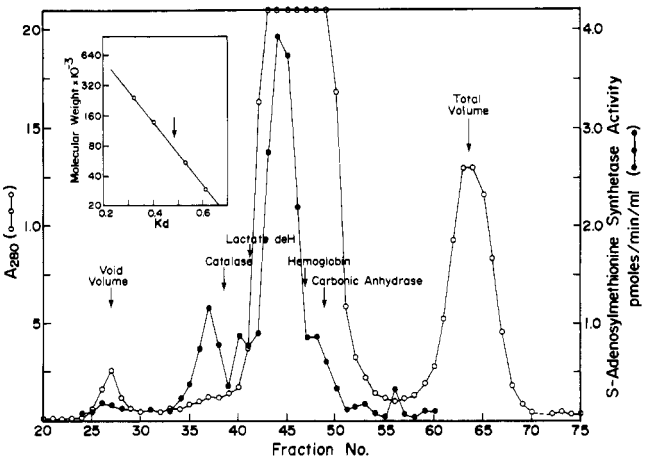

FIGURE 9: Gel filtration chromatography of red cell cytosol on Sephacryl S-300. Red cell cytosol (180 mg of protein) was fractionated, and *S*-adenosylmethionine synthetase and marker proteins were assayed as described under Methods and Materials. Absorbance at 280 nm is indicated (O). The distribution coefficients K_d [(peak volume − void volume)/(total volume − void volume)] of the marker proteins are shown in the inset. Molecular weight values used were the following: catalase, 240 000; lactate dehydrogenase, 140 000; hemoglobin, 55 000; carbonic anhydrase, 29 500. The major peak of *S*-adenosylmethionine synthetase activity has a K_d of 0.48, while the smaller peak of activity has a K_d of 0.28. From these data, approximate molecular weights of 74 000 and 320 000 were obtained, respectively.

Discussion

Human Erythrocyte S-Adenosylmethionine Synthetase: A Single Enzyme Species. The intracellular concentration of *S*-adenosylmethionine in the erythrocyte is determined by the relative rates of its biosynthesis and its utilization by methyltransferases and other enzymes. We have characterized the biosynthetic enzyme and report here kinetic data which clarify the role of this enzyme in maintaining the level of *S*-adenosylmethionine. We find no evidence in the red cell for the presence of isozymes such as those described in liver. For example, rat liver has at least two (and possibly three) major *S*-adenosylmethionine synthetase activities which can be chromatographically separated and distinguished by their kinetic properties, especially with regard to the affinity for methionine (Table V). However, the human erythrocyte enzyme fractionates as one major species on both ion-exchange chromatography (Figure 4) and gel filtration chromatography (Figure 9). The kinetic data obtained here are also consistent

Table V: Summary of Properties of *S*-Adenosylmethionine Synthetases

properties	red cell[a]	rat liver[b] I	rat liver[b] II	rat liver[b] III	rat liver[d,e] α	rat liver[d,e] β	rat kidney[d] γ	yeast[f]	*E. coli*[g]
K_m(methionine) (μM)	2.2 ± 0.6	30–45	6	200–300[c]	17[c]	500[c]	6	110–140[c]	100
K_m(ATP) (μM)	450 ± 130				500[c]	2000[c]	70	47–74[c]	130
K_i(*S*-adenosylmethionine) (μM)	2.9 ± 0.5	400	65	activation					10–60
approximate native molecular weight (×10³)	74	210	190	170	210	100[e]–160[d]	190	110	180
specific activity in crude extract [pmol min⁻¹ (mg of protein)⁻¹]	0.05–0.2			75.5[d,h]			32	670	210

[a] This study. [b] Hoffman & Sullivan (1981); Hoffman & Kunz (1980). [c] Apparent value; non Michaelis–Menten kinetics. [d] Okada et al. (1981). [e] Abe et al. (1982). [f] Chiang & Cantoni (1977). [g] Markham et al. (1980). [h] Assayed with 25 μM methionine.

with the presence of a single enzyme species, most notably in the observation of a single K_m value for methionine both in a dialyzed cytosolic preparation and in a 350-fold purified enzyme preparation (Figures 1 and 5).

Comparison of the Enzymatic Properties of Dialyzed Cytosol and of a Partially Purified Enzyme Preparation. To understand the regulation of erythrocyte *S*-adenosylmethionine synthetase activity, we analyzed kinetic data obtained in a cytosolic preparation extensively dialyzed to remove small molecules. In this extract endogenous protein factors would be present which may play a role in the catalytic or regulatory properties of this enzyme. We compared the properties of this preparation with those of a 350-fold purified preparation. Here, many enzymes which may bind and/or transform the substrates and products of the adenosylmethionine synthetase reaction are removed. We find that the values for the K_m of methionine and the K_i for *S*-adenosylmethionine are nearly identical in both enzyme preparations (Figures 1 and 5). On the other hand, the activation by Mg^{2+} and the apparent K_m for Mg·ATP differ considerably. At a physiological level of 1 mM ATP, an optimal $MgCl_2$ concentration of 20 mM or higher is found for the cytosolic enzyme, whereas 5 mM $MgCl_2$ is optimal for the partially purified enzyme. Since we have not measured the actual free Mg^{2+} and Mg·ATP concentrations in each case, it is possible that the removal of free Mg^{2+} by protein binding may necessitate the higher level of this ion required to fully activate cytosolic adenosylmethionine synthetase. The differences in the apparent K_m for ATP under optimal $MgCl_2$ levels are also significant in the two enzyme preparations. A value of 80 μM has been measured in the crude extract (Figure 3), whereas the value for the purified enzyme is 450 μM (Figure 8). It is thus possible that some protein factor in the cytosol may increase the affinity of the enzyme for ATP. We have considered the possibility that this factor is calmodulin, but the enzymatic activity in the cytosol is not affected by the addition of either 1 mM $CaCl_2$ or 1 mM EDTA. Another possibility is that the enzyme is regulated by covalent modification and that the degree of this modification is different in the cytosol and in the purified enzyme fraction.

Regulation of S-Adenosylmethionine Levels in Red Cells by Substrate and Product Concentrations. The concentrations of the substrates and products of this enzyme in the erythrocyte are given in Table IV. When these levels are compared to the kinetic values measured, we conclude that small changes in the concentration of three of these metabolites can affect the enzyme activity, while changes in two of these levels will probably have no effect. For example, the low K_m for methionine (2.2 μM) and the larger physiological concentration of methionine (12–24 μM) indicate that the enzyme is generally saturated with this substrate. Increases in methionine concentration would thus not lead to greater activity. It has been found that red cell *S*-adenosylmethionine levels are not markedly altered by oral feeding of large doses of methionine in rats and humans (Baldessarini et al., 1978). On the other hand, oral feeding or perfusion of methionine increases *S*-adenosylmethionine levels in rat liver from 5- to 7-fold (Hoffman et al., 1980; Schatz et al., 1981). This latter effect is probably due to the presence of an adenosylmethionine synthetase isozyme in liver (form III/β, Table V) with a high K_m for methionine.

It has been shown that increasing levels of the product of transmethylation reactions, *S*-adenosylhomocysteine, increase *S*-adenosylmethionine levels in liver, and it has been proposed that *S*-adenosylhomocysteine may be a positive regulator of liver *S*-adenosylmethionine synthetase (Hoffman et al., 1980). This does not appear to be the case with the erythrocyte enzyme. At or above the physiological level of adenosylhomocysteine measured here (1.3 μM), little or no effect is observed on the activity (Figures 1 and 5).

On the other hand, changes in the concentration of Mg^{2+}, ATP, and *S*-adenosylmethionine do appear to affect the red cell enzyme activity at physiological levels. The most marked effect is the product inhibition seen with *S*-adenosylmethionine (K_i = 2.0–2.9 μM; Figures 1 and 5). Since the concentration of *S*-adenosylmethionine in the erythrocyte is slightly larger than this inhibition constant (Table IV), the intracellular concentration of this metabolite will be effectively buffered around this value. The degree of product inhibition seen here appears to be much greater than that observed with other enzymes (Table V), and this may reflect the fact that *S*-adenosylmethionine concentrations are generally much higher in nonerythrocyte cells (Eloranta, 1977).

The red cell enzyme may also be regulated by Mg^{2+} and ATP levels. The purified enzyme has a K_m for Mg·ATP of slightly less than the physiological concentration (Figure 8 and Table IV). Of perhaps more importance is the fact that the enzyme appears to be activated by free Mg^{2+} (Figures 2 and 6). The nature of this activation is complex. Calculations of the concentrations of Mg·ATP, Mg_2·ATP, and free Mg^{2+} as described (Kohlbrenner & Cross, 1979; Storer & Cornish-Bowden, 1976) reveal that either of the latter two species may be required for full activity. Whether free Mg^{2+} or the Mg_2·ATP complex functions as substrates or as allosteric activators (perhaps by stabilizing a high-affinity form of the enzyme; cf. Figure 8) is not clear from the present data. It is interesting that the *S*-adenosylmethionine synthetase from *E. coli* does show a requirement for an additional Mg^{2+} ion in addition to the Mg·ATP complex for full activity (Markham, 1981).

Comparison of the Human Erythrocyte S-Adenosylmethionine Synthetase with Enzymes from Other Cell Types: A Distinct Red Cell Enzyme. Table V summarizes the properties of two purified *S*-adenosylmethionine synthetases from *E. coli* and yeast with those of several partially to fully purified mammalian species. It is apparent that each of these enzymes differs in its kinetic properties and molecular weight. For example, the rat liver isozyme III is activated by product while the human red cell enzyme, the rat liver isozyme II, and the *E. coli* enzyme show varying degrees of product inhibition with *S*-adenosylmethionine. In each of the latter cases, the K_i values for *S*-adenosylmethionine inhibition are similar to the intracellular concentrations of *S*-adenosylmethioinine in these tissues and suggest that this kinetic property may be important in the physiological regulation of the level of this metabolite. Among the mammalian enzymes, the isozyme III/β of liver appears to be specialized for a catabolic function in the conversion of excess dietary methionine into cysteine via *S*-adenosylmethionine (Hoffman et al., 1980). This enzyme is not subject to product inhibition by *S*-adenosylmethionine and has a very large K_m for methionine.

The *S*-adenosylmethionine synthetase of erythrocytes thus appears to be a new type of enzyme, characterized by its high affinity for its substrate methionine and its product *S*-adenosylmethionine and its relatively low molecular weight. The activity of this enzyme measured here in the red cell cytosol is much less than that of crude extracts of other cell types (Table V). This is consistent with the lower level of *S*-adenosylmethionine in erythrocytes. To ensure that no procedural error was involved in our assay, we measured the

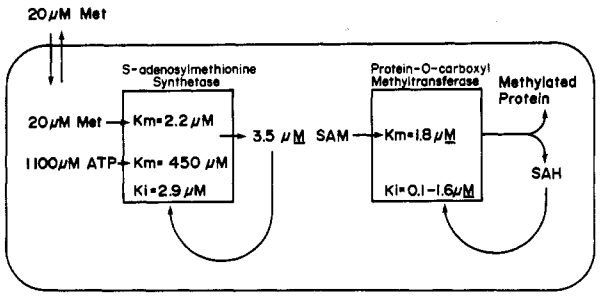

FIGURE 10: Metabolic regulation of *S*-adenosylmethionine synthesis and protein methylation in human erythrocytes. Approximate physiological concentrations of metabolites are shown (see Table IV). The K_m and K_i values for *S*-adenosylmethionine synthetase were determined from this work. The K_m for *S*-adenosylmethionine (SAM) for the protein methyltransferase has been determined as 1.8 μM (Kim, 1974); the K_i for *S*-adenosylhomocysteine (SAH) for this enzyme has been given as 0.1 (Gillet et al., 1979) or 1.6 μM (Kim, 1974).

activity of the enzyme in rat liver cytosol and obtained similar specific activities to those reported in the literature (Okada et al., 1981).

Role of S-Adenosylmethionine in Erythrocytes. Although *S*-adenosylmethionine participates in a variety of transmethylation reactions in most cells, as well as serving as a precursor for polyamine synthesis, its role in the red cell appears to be much more limited. Part of this reflects the simplicity of the erythrocyte. For example, nucleic acids, substrates for several types of transmethylation reactions, are essentially absent from red cells, and polyamine synthesis has not been observed. Many of the common transmethylation reactions do not occur to any appreciable extent. In fact, when red cell are incubated with [*methyl*-^{3}H]methionine, the bulk of the radioactivity incorporated into the membrane fraction is in the form of protein methyl esters (Freitag & Clarke, 1981). Less than 2% of the radioactivity is incorporated into lipids, and nonprotein components account for less than 10% of the total. The only other transmethylation reactions which have been proposed for red cells are based on the detection of catechol *O*-methyltransferase and histamine *N*-methyltransferase activity (Axelrod & Cohn, 1971; Quiram & Weinshilboum, 1976). It is not clear whether these reactions occur in vivo to any appreciable extent. Therefore, it appears that the bulk of the pool of red cell *S*-adenosylmethionine may be maintained for the single reaction of the carboxyl methylation of membrane and cytosolic proteins (Freitag & Clarke, 1981; McFadden & Clarke, 1982; Figure 10). Thus the level of *S*-adenosylmethionine in erythrocytes may be maintained at a relatively low level, probably largely by product inhibition, that is nevertheless sufficient for this protein methylation reaction.

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Registry No. ATP, 56-65-5; Mg·ATP, 1476-84-2; L-methionine, 63-68-3; magnesium, 7439-95-4; *S*-adenosylhomocysteine, 979-92-0; *S*-adenosylmethionine, 29908-03-0; *S*-adenosyl-L-methionine synthetase, 9012-52-6.

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6-(*p*-Toluidinyl)naphthalene-2-sulfonic Acid as a Fluorescent Probe of Yeast Hexokinase: Conformational States Induced by Sugar and Nucleotide Ligands†

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ABSTRACT: The fluorescent dye 6-(*p*-toluidinyl)naphthalene-2-sulfonic acid (2,6-TNS) has been shown to be a sensitive and nonperturbing probe of conformational states of yeast hexokinase. The binding of sugar ligands to hexokinase induced conformational states of the enzyme which could be distinguished by monitoring 2,6-TNS fluorescence and correlated well with their behavior during the catalytic reaction. The binding of five-carbon sugar inhibitors such as lyxose induced a conformational state of hexokinase that demonstrated a small quenching of 2,6-TNS fluorescence but an increased ability to bind metal–ligands when compared to free enzyme. The binding of good sugar substrates such as glucose produced a conformational state of hexokinase which demonstrated a large enhancement (37%) of bound 2,6-TNS fluorescence. This glucose-induced conformational state had an increased ability to bind metal–ATP ligands; however, the relative changes in the dissociation constants for the various metal–ATP ligands differ from those observed with hexokinase in the presence of lyxose. Hence, the lyxose-induced conformational state of hexokinase was concluded to be significantly different from the glucose-induced conformational state. The binding of poor sugar substrates such as 5-thioglucose induced a conformational state of hexokinase similar to the conformational state induced by glucose, but with a smaller enhancement of 2,6-TNS fluorescence (15%) and a lesser ability to increase the affinity for metal–ATP ligands. The six-carbon inhibitor with a bulky group on the 2-position, *N*-acetylglucosamine, gave minimal changes in 2,6-TNS fluorescence and effects on metal–nucleotide binding. These conformational states are interpreted in terms of the closure of the cleft between the two domains observed by X-ray crystallography. The binding of AlATP to free hexokinase was not observed at concentrations up to 100 μM, which is consistent with the kinetic properties reported for this metal–ATP ligand. Although both CrATP and AlATP have been reported to produce a slow burst-type transient in the progress curve of hexokinase, only CrATP demonstrated slow changes in 2,6-TNS fluorescence, indicating that the conformational state of hexokinase induced by AlATP is different from the conformational state induced by CrATP.

Yeast hexokinase catalyzes the phosphorylation of certain hexose sugars in the presence of MgATP. The rate at which this reaction proceeds varies among the sugars from good substrates such as glucose to poor substrates such as 5-thioglucose and mannosamine (De Domenech & Sols, 1980; Viola et al., 1980). Metal(III)–ATP ligands act as inhibitors of hexokinase, and several of these ligands cause a slow, burst-type transient in the hexokinase progress curve when added to the assay mixture (Danenberg & Cleland, 1975; Viola et al., 1980). A random mechanism for the hexokinase-catalyzed phosphorylation reaction has been demonstrated (Fromm & Zewe, 1962; Rudolph & Fromm, 1971b; however, a preferred order of substrate addition, with sugar substrates binding prior to metal–nucleotide, has also been reported (Noat et al., 1969; Wilkinson & Rose, 1979; Danenberg & Cleland, 1975). Recent kinetic studies have demonstrated synergism in the binding of MgATP and certain sugar substrates (Viola et al., 1980, 1982). Several investigators have suggested that substrate–hexokinase interactions involve conformational changes in the enzyme. Physical studies of hexokinase structure, including X-ray crystallography (Bennett & Steitz, 1978), X-ray light scattering (McDonald et al., 1979), and intrinsic fluorescence (Zewe et al., 1964; Peters & Neet, 1978), have demonstrated that a large conformational change in hexokinase occurs upon binding glucose. X-ray crystallography suggests that the large conformational change with glucose is a movement of two domains of the enzyme toward each other with glucose situated within the cleft (Bennett & Steitz, 1978); this closure is sterically inhibited by certain six-carbon inhibitors with bulky groups in the 2-carbon position, such as *o*-toluoylglucosamine (Anderson et al., 1978). Furthermore, the binding of xylose to hexokinase crystals induced structural changes that were different from those observed upon binding glucose or *o*-toluoylglucosamine (Anderson & Steitz, 1975). The binding of glucose to yeast hexokinase results in a 12% quenching of intrinsic tryptophan fluorescence (Zewe et al., 1964; Feldman & Kramp, 1978) which is independent of ionic strength (Mayer et al., 1982). Fluorescence titration experiments have shown that glucose enhanced the binding of Cr$(NH_3)_2$ATP and that lyxose, a five-carbon analogue of glucose, quenched the intrinsic fluorescence of yeast hexokinase only after prior addition of Cr$(NH_3)_2$ATP (Peters & Neet, 1978).

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