Comparison of *S*-Adenosylmethionine Decarboxylases from Rat Liver and Muscle†

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ABSTRACT: *S*-Adenosylmethionine decarboxylase was purified to homogeneity from rat liver and from rat psoas. The major step involved affinity chromatography on methylglyoxal bis(guanylhydrazone) linked to Sepharose. The muscle enzyme was more tightly retained to this absorbent, and the enzymes from the two sources could readily be separated by chromatography on this material. The psoas and liver enzymes could not be distinguished by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, both giving a single band corresponding to an M_r of 32 500, but were separated by electrophoresis under nondenaturating conditions and by isoelectric focusing (the isoelectric points were 5.3 for psoas and 5.7 for liver enzyme). The liver and psoas enzymes also differed in respect to K_m for *S*-adenosylmethionine, the degree to which they were activated by putrescine, and their sensitivity to inhibition by methylglyoxal bis(guanylhydrazone) and related compounds. These results indicate that there are two forms of *S*-adenosylmethionine decarboxylase. The presence of a particular form could, therefore, be important both in the regulation of polyamine levels and also in the pharmacology involving inhibitors of polyamine synthesis.

The aminopropyl moieties of spermidine and spermine are derived from methionine in mammalian cells (Tabor & Tabor, 1976; Jänne et al., 1978; Williams-Ashman & Pegg, 1981). The immediate aminopropyl donor is decarboxylated *S*-adenosylmethionine, and the enzyme responsible for this decarboxylation has been extensively purified from rat liver and prostate (Pegg, 1974, 1977, 1979; Demetriou et al., 1978) and from mouse liver and mammary gland (Sakai et al., 1979). The enzyme is located in the cytosol and is activated by putrescine (Pegg & Williams-Ashman, 1969). It is inhibited by the drug methylglyoxal bis(guanylhydrazone) (MGBG)[1] (Williams-Ashman & Schenone, 1972). In a recent review, Williams-Ashman & Pegg (1981) pointed out that at the time of writing there was no convincing evidence for multiple forms of this enzyme. Sturman (1976a,b) had observed an activity liberating CO_2 from the carboxyl group of *S*-adenosylmethionine in detergent-treated rat liver particulate extracts, but subsequent investigations revealed that this activity was the result of a number of reactions degrading *S*-adenosylmethionine and releasing CO_2 from some of the products (Eloranta & Raina, 1978; Wilson et al., 1979; Pegg, 1979). Nevertheless, the possibility that different forms of the true *S*-adenosylmethionine decarboxylase exist had not been extensively tested, and recent experiments in this laboratory suggested that there may be differences between the enzymes present in liver and diaphragm or psoas muscle (Pösö & Pegg, 1981a,b). Specifically, we noted that the enzyme in liver was less sensitive to inhibition after injection of spermidine than the enzyme in diaphragm or psoas. Also, the stimulation of activity by putrescine was greater in dialyzed extracts from these muscles than in similar extracts from liver (Pösö & Pegg, 1981b). All of these comparisons were made by using crude tissue homogenates, and many possible explanations for the results other than the existence of different forms of the enzyme were possible. Therefore, in the experiments described in the present paper, we purified the enzyme to homogeneity from liver and psoas and compared the properties of the enzymes directly. The enzymes from liver and muscle were different in their sensitivity to inhibitors, activation by putrescine, and K_m for *S*-adenosylmethionine. The enzymes could be separated by chromatography on MGBG–Sepharose and by polyacrylamide gel electrophoresis, and they had different isoelectric points. There is considerable interest in the polyamine biosynthetic pathway as a target for inhibitors having chemotherapeutic potential. The presence of different forms of *S*-adenosylmethionine decarboxylase may provide an opportunity for selective alteration of polyamine production in various tissues.

Experimental Procedures

Materials. *S*-Adenosyl-L-[*carboxy*-^{14}C]methionine (54.2 Ci/mol) was purchased from New England Nuclear, Boston, MA. Unlabeled *S*-adenosylmethionine was obtained from Sigma Chemical Co., St. Louis, MO, and purified prior to use (Pegg & Williams-Ashman, 1969). MGBG was purchased from Aldrich Chemical Co., Milwaukee, WI. MGBG–Sepharose was prepared as described by Pegg (1974). Ultrogel AcA34 was obtained from LKB, Rockville, MD. All other reagents were obtained from Sigma or from Pharmacia Fine Chemicals, Piscataway, NJ. Congeners of MGBG were generous gifts from Dr. E. Mihich, Roswell Park Memorial Institute, Buffalo, NY.

Enzyme Assays. *S*-Adenosylmethionine decarboxylase was assayed as previously described (Pegg, 1974), and one unit of activity was defined as the release of 1 nmol of CO_2/min at 37 °C. Assays were routinely carried out for 30 min at 37 °C except where otherwise indicated. All assays were performed under conditions where activity was proportional to the protein added and the time of incubation. Protein was determined by the method of Bradford (1976) or by measurement of the absorbance at 260 and 280 nm (Warburg & Christian, 1941).

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[1] Abbreviations: MGBG, methylglyoxal bis(guanylhydrazone); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Purification of *S*-Adenosylmethionine Decarboxylase from Rat Liver and Psoas[a]

tissue	fraction	total protein (mg)	total units	sp act. (units/mg)	*x*-fold purification	yield (%)
liver	supernatant	21352	1600	0.08[b]	1	100
liver	dialyzed $(NH_4)_2SO_4$	8160	1683	0.21[b]	3	105
liver	DEAE-cellulose eluate	259	1203	4.60	61	75
liver	MGBG–Sepharose eluate	1.1	695	632	8427	43
psoas	supernatant	4459	178	0.04[a]	1	100
psoas	dialyzed $(NH_4)_2SO_4$ precipitate	991	158	0.16[a]	4	89
psoas	DEAE-cellulose eluate	35	107	2.90	72	57
psoas	MGBG–Sepharose eluate	0.14	87	644	16100	49

[a] The rats were pretreated with 80 mg/kg MGBG 22–24 h before death, and livers and psoas from about 35 animals were used. One unit of enzyme released 1 nmol of CO_2/min at 37 °C. [b] Measured in sample aliquots freed from residual MGBG by dialysis.

Enzyme Purification. The procedure used was as described by Pegg (1977) with the exception that the MGBG–Sepharose chromatography was varied by washing the column after the enzyme was attached with 250 mL of 10 mM Tris-HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 0.5 M NaCl. This increase in NaCl concentration from 0.3 to 0.5 M removed more contaminating proteins, and the enzyme was not eluted until 1 mM MGBG was added to the eluting solution. The concentration of MGBG in the column eluate was determined from the absorbance at 283 nm by using a molar extinction coefficient of 38 400 at pH 1 (Pegg, 1974). With this procedure, it was not necessary to pass the enzyme through the column twice to obtain a homogeneous preparation, as with earlier preparations using a lower salt concentration (Pegg, 1974, 1977, 1979).

The enzyme was purified from the liver and psoas obtained from 35 male Sprague-Dawley rats weighing 400–500 g which were treated with 80 mg/kg MGBG by intraperitoneal injection 22–24 h prior to death to increase the amount of enzyme (Pegg, 1974). The tissues were homogenized with 2 volumes of ice-cold 25 mM sodium phosphate, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM putrescine, and 0.1 mM EDTA (buffer A). All steps were carried out at 0–4 °C. The homogenate was centrifuged at 105000*g* for 30 min and the supernatant fractionated by addition of ammonium sulfate. Proteins precipitating between 35% and 65% saturation with ammonium sulfate were collected by centrifugation at 10000*g*, dissolved in 100 mL of buffer A, and dialyzed for 18 h against 5 L of buffer A. The dialyzed solution (about 200 mL) was then applied to a column (5 cm × 45 cm) of DEAE-cellulose equilibrated with buffer A without EDTA (buffer B). The column was then washed with 200 mL of 0.1 M NaCl in buffer B and eluted with a linear gradient of 0.1–0.3 M NaCl in buffer B (2 L total volume) at a flow rate of 80 mL/h. Fractions of 20 mL volume were collected, and those containing enzyme activity were pooled (enzyme is eluted at about 0.18 M NaCl) and made 70% saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation at 10000*g*, dissolved in 40 mL of 10 mM Tris-HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM dithiothreitol, and 0.1 mM EDTA (buffer C), and dialyzed against 4000 mL of buffer C for 15 h. The extract was applied to a column (1.6 cm × 18 cm) of MGBG–Sepharose in buffer C. [MGBG–Sepharose was prepared as described by Pegg (1974, 1977).] The column was loaded and run at a flow rate of 45 mL/h. After the column was washed with 200 mL of buffer C, followed by 200 mL of buffer C containing 0.5 M NaCl, the *S*-adenosylmethionine decarboxylase was eluted by buffer C containing 0.5 M NaCl and 1 mM MGBG. Fractions of 10 mL were collected and assayed for activity after dialysis (for 6 h against buffer C) or dilution of aliquots (at least 50-fold with buffer C) to reduce the inhibitor concentrations to levels which did not interfere with the assay. The concentration of MGBG in the eluate was determined from the absorbance at 283 nm by using a molar extinction coefficient of 38 400 at pH 1 (Pegg, 1974). The fractions containing enzyme were concentrated by ultrafiltration to a volume of about 1 mL. About 10 mL of buffer C was then added and the solution concentrated again. This procedure was repeated 5 times to remove MGBG. Any remaining traces were removed by dialysis for 48 h against 1 L of buffer C. The final preparation showed no trace of MGBG when the absorbance spectrum was measured and gave activities proportional to the amount of protein added when assayed in the standard assay. A representative purification is given in Table I. Other preparations gave similar yields and final specific activities. The enzyme was stored at a concentration of about 1 mg/mL for the liver enzyme and about 0.2 mg/mL for the psoas enzyme dissolved in 10 mM Tris-HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM dithiothreitol, and 0.1 mM EDTA and was stable for at least 6 weeks at 0–2 °C. About 40–50% of the activity was lost in 1 week at −25 °C.

Electrophoresis. Disc polyacrylamide gel electrophoresis of the native enzymes was performed at either pH 7.2 or pH 8.5 (Ornstein, 1964) and of the denatured enzyme in the presence of sodium dodecyl sulfate (Laemmli, 1970). Isoelectric focusing was carried out according to the method described by the manufacturer by using LKB Ampholine PAG plates and a LKB 2103 apparatus (LKB, Rockville, MD). The enzyme preparations were separated by using LKB 1804-102 gel (pH range 4–6.5), a cathode electrode solution of 0.1 M β-alanine, and an anode electrode solution of 0.1 M glutamic acid in 0.5 M H_3PO_4. The plates were run with a voltage of 1580 V and current of 15 mA for 135 min at 10 °C. The pH gradient at the end of the run was checked by using a surface pH electrode. Protein was then stained with Coomassie brilliant blue.

Results

When rats were pretreated with MGBG, there was a substantial increase in *S*-adenosylmethionine decarboxylase activity in rat liver as previously reported (Pegg, 1974) and in psoas. The increase was about 20-fold in liver and 35-fold in psoas. Tissues taken from rats treated in this way were used for the purification of the enzyme (Table I) by a slight modification of the method previously used for rat liver (Pegg, 1974, 1977). After fractionation with ammonium sulfate and chromatography on DEAE-cellulose, the enzyme was purified by affinity chromatography on MGBG–Sepharose. All of the enzyme applied to this column was retained and remained attached even when the washing buffer contained 0.5 M NaCl. After other proteins were washed off by this solution, the enzyme was eluted by addition of 1 mM MGBG. As can be seen from Table I, this method was also appropriate for the

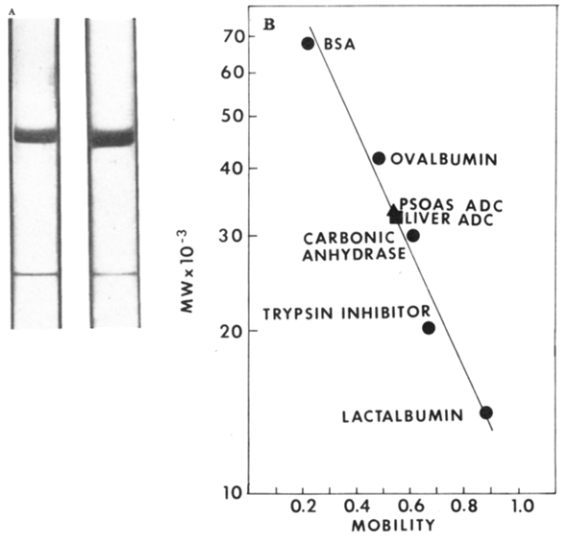

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of liver and psoas *S*-adenosylmethionine decarboxylases. The enzymes were separated on 7.5% gels and stained with Coomassie blue. Figure 1A shows results for somewhat overloaded gels (15 μg) to indicate the absence of other components. The direction of migration is from top to bottom. The gel on the left is enzyme from psoas and that on the right from liver. Figure 1B shows the relative mobilities of the enzyme bands compared to known protein markers.

enzyme from muscle; from both sources, material with a similar specific activity was obtained in 40–50% yield.

The enzyme purified in this way gave a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1A), and the mobilities of the bands from both liver and psoas were similar and corresponded to an M_r of about 32 500 (Figure 1B). When chromatographed on Ultrogel AcA34, the native enzyme from both sources eluted as a single peak in identical fractions corresponding to an apparent M_r of 68 000, suggesting that the enzymes have two subunits (results not shown). When the same enzyme preparations were subjected to electrophoresis on nondenaturing polyacrylamide gel electrophoresis and the gels tested for enzyme activity and the presence of protein, the liver enzyme gave two bands which stained with dye (Figure 2A, left) and both had activity (Figure 2B, middle panel). The slower moving band was rather variable from experiment to experiment and was greatest in experiments in which larger amounts of enzyme were loaded onto the gels. It could be due to aggregation since liver *S*-adenosylmethionine decarboxylase is known to form larger aggregates quite readily (Demetriou et al., 1978). The enzyme from psoas gave a single protein band (Figure 2A, center) which corresponded to the activity (Figure 2B, middle panel). A mixture of the two enzymes gave the expected three bands (Figure 2A, right) and peaks of activity (Figure 2B, top panel).

A consistent finding during the purification of the enzymes in the affinity chromatography step was that the enzyme from psoas was eluted in a later fraction (40–50 mL) than that from liver, which was always eluted between 20 and 30 mL after the addition of MGBG to the elution buffer. This also suggests a difference between the enzymes, but it was possible that it was due to the fact that much more protein and more enzyme activity was applied to the same size column when the liver enzyme was being purified. Therefore, equal amounts of the liver and psoas enzymes were taken, combined, and applied to the MGBG–Sepharose column. As shown in Figure 3, lower panel, the activity eluted in two separate peaks. The first of these, peak A, corresponded to the position seen when the liver enzyme was purified, and when peak A was concentrated and run on polyacrylamide gel electrophoresis, two peaks of activity

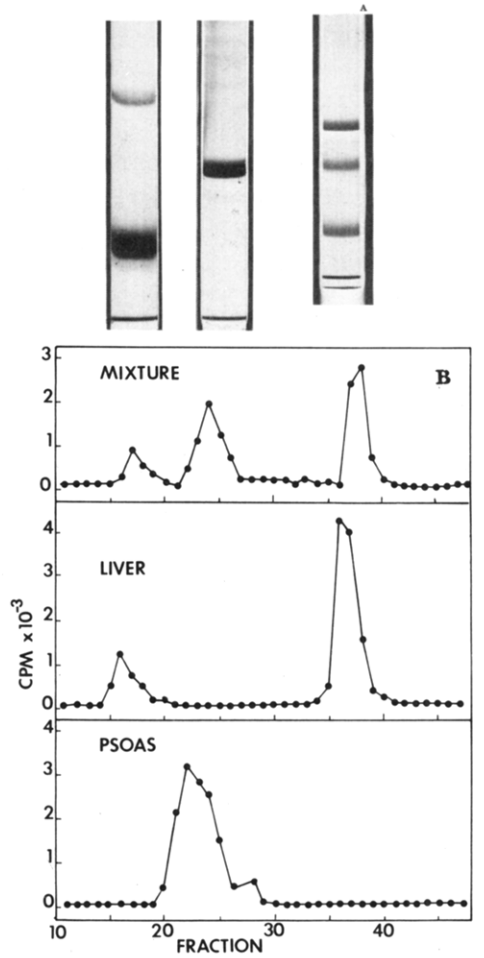

FIGURE 2: Polyacrylamide gel electrophoresis of native liver and psoas *S*-adenosylmethionine decarboxylases. Electrophoresis was carried out on 5% gels (0.6 × 11 cm slab gels) in 25 mM Tris-HCl, 190 mM glycine, 5 mM dithiothreitol, and 2.5 mM putrescine, pH 8.5, at 5 mA per gel at 4 °C for 12 h. Figure 2A shows results for 12 μg of liver enzyme (left), 8 μg of psoas enzyme (center), and a mixture of 16 μg of liver and 8 μg of psoas enzymes (right). The separate liver and psoas enzymes were run at the same time and correspond to the activity measurements in Figure 2B. The mixture was run in a different experiment. Gels were stained with Coomassie blue. Figure 2B shows enzyme activity eluted from gels. Results are shown for enzyme from psoas (8 μg; lower panel), liver (12 μg; middle panel), and a mixture of the two with 6 μg from psoas and 10 μg from liver (upper panel). After electrophoresis, the gels were sliced into 2-mm-thick sections, and the enzyme was eluted by shaking overnight in 0.5 mL of 25 mM sodium phosphate, 0.1 mM EDTA, 2.5 mM dithiothreitol, and 2.5 mM putrescine, pH 7.5 at 4 °C. The enzyme activity was then determined by adding 0.2 μCi of *S*-adenosyl-[*carboxy*-^{14}C]methionine, incubating at 37 °C for 90 min, and measuring $^{14}CO_2$ production.

corresponding to those of the purified liver enzyme were seen (Figure 3, middle panel). The second peak, B, corresponded to the position seen when the psoas enzyme was purified and when run on polyacrylamide gel electrophoresis gave the typical peak of the muscle enzyme intermediate between the liver enzyme peaks (Figure 3, upper panel).

S-Adenosylmethionine decarboxylases from liver and psoas could also be separated from each other by electrophoresis on isoelectric focusing gels. Each enzyme gave a single band in such gels corresponding to an isoelectric point of 5.3 for psoas enzyme and 5.7 for the enzyme from liver (Table II).

The purified *S*-adenosylmethionine decarboxylases from psoas and liver were compared with respect to activation by putrescine, the K_m for *S*-adenosylmethionine in the presence of a maximally stimulating concentration of putrescine, and

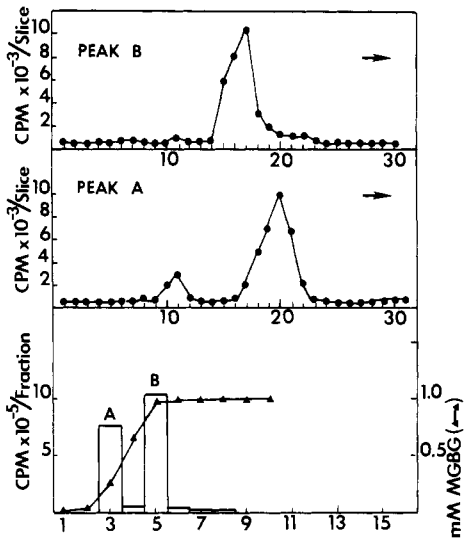

FIGURE 3: Separation of liver and muscle *S*-adenosylmethionine decarboxylases by chromatography on MGBG–Sepharose. Equal amounts of purified *S*-adenosylmethionine decarboxylase from liver and psoas were mixed together and applied to a MGBG–Sepharose column. The column was washed with 250 mL of the buffer containing 0.5 M NaCl as described under Experimental Procedures, and then 1 mM MGBG was introduced into the elution buffer. The lower panel shows the fractions containing activity and the MGBG concentration in the eluate (measured by the extinction at 283 nm). The middle panel shows the results of polyacrylamide gel electrophoresis of the fraction A eluted in fraction 3 and the upper panel that of fraction B eluted in fraction 5. Electrophoresis was carried out on 7.5% gels in 50 mM sodium phosphate, 5 mM dithiothreitol, and 2.5 mM putrescine at 4 °C for 16 h. Other details and the measurement of enzyme activity were as in Figure 2.

Table II: Comparison of Liver and Psoas *S*-Adenosylmethionine Decarboxylases

source of enzyme	isoelectric point	K_m for *S*-AdoMet[a] (μM)	K_a for putrescine[b] (μM)	*x*-fold max stimulation by putrescine
liver	5.7	50	25	6
psoas	5.3	100	28	15

[a] The K_m for *S*-adenosylmethionine was determined in the standard assay solution containing 2.5 mM putrescine and about 1 μg of protein. [b] The activation by putrescine was studied by using enzyme freed from putrescine by dialysis for 14 h at 0–4 °C against two changes of 4 L of 25 mM sodium phosphate, 2.4 mM dithiothreitol, and 0.1 mM EDTA, pH 7.5. The assays contained varying amounts of putrescine and 1.4 μg of protein and were incubated at 37 °C for only 10 min to ensure that linear rates of reaction were achieved when little or no putrescine was present. The enzyme is very unstable in the absence of putrescine.

inhibition by MGBG and various congeners. The purified enzymes were stimulated by putrescine, both had an apparent K_a of 25–30 μM, and both required 0.25 mM for maximal activation. However, at this concentration, the psoas enzyme activity was enhanced about 15-fold whereas the liver enzyme activity was increased only 6-fold (Table II). These results were for assays at pH 7.5. Activation by putrescine is known to be pH dependent, being greatest at lower pH values (Pegg & Williams-Ashman, 1970; Corti et al., 1974; Williams-Ashman & Pegg, 1981), and the assays were also conducted at pH 6.5 and pH 8.5 (results not shown). Although the magnitude of the stimulation varied in accordance with the pH as previously reported (Pegg & Williams-Ashman, 1970; Corti et al., 1974), in all cases the muscle enzyme was stimulated 2–3 times more than the liver enzyme. These results cannot be explained by the presence of putrescine in the liver enzyme preparation because removal was assessed by adding

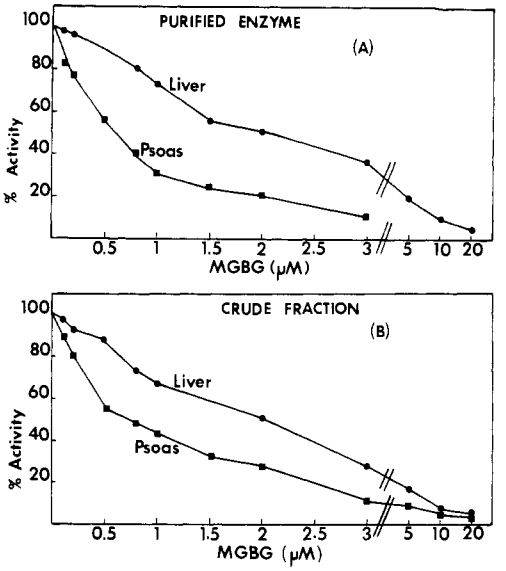

FIGURE 4: Inhibition of *S*-adenosylmethionine decarboxylase by MGBG. The enzyme was assayed in the presence of 0.2 mM *S*-adenosylmethionine and 2.5 mM putrescine in 25 mM Tris-HCl and 0.1 mM EDTA, pH 7.5. Results are shown in Figure 4A for highly purified liver (●) or psoas enzyme (■) and in Figure 4B for crude fractions from liver (●) or psoas (■). In both cases, results were expressed as percentages of the activity seen in the absence of MGBG for assay at the concentrations of MGBG shown. The assays of purified enzymes were carried out as in Table II. Assays of the crude fraction (dialyzed ammonium sulfate precipitate) contain 750 μg of protein and were carried out for 30 min at 37 °C. At least 400 cpm above background was released in all assays. These experiments and those in Table III were carried out in Tris-HCl buffer which is weakly inhibitory to *S*-adenosylmethionine decarboxylase (Pegg & Williams-Ashman, 1969) in order to avoid any possible precipitation of MGBG congeners in phosphate buffer. Exactly the same degree of inhibition by MGBG was seen when phosphate buffer was used for this experiment.

labeled putrescine to the stored enzyme and there was no difference in the radioactivity present in the two enzyme preparations after dialysis.

S-Adenosylmethionine decarboxylase activity in crude enzyme preparations can also be stimulated by other diamines such as 1,3-diaminopropane and to a very small extent by spermidine (Pegg & Williams-Ashman, 1970; Williams-Ashman & Schenone, 1972; Corti et al., 1974; Sakai et al., 1979; Mamont & Danzin, 1981). The enzyme from psoas was stimulated more by 1,3-diaminopropane than the enzyme from liver (1 mM diaminopropane stimulated 4-fold at pH 6.5 as opposed to 2-fold for the liver enzyme). The stimulation of the purified enzyme by spermidine was very small, amounting to less than 50% for both enzymes when 1 mM spermidine was present (results not shown).

The apparent K_m for *S*-adenosylmethionine measured at pH 7.5 in the presence of a saturating amount of putrescine was also different for the two enzymes (Table II). The K_m with the liver enzyme was about 50 μM, and that for the enzyme from psoas was 100 μM.

The purified enzymes also differed in sensitivity to MGBG when tested at saturating *S*-adenosylmethionine and putrescine concentrations (Figure 4A). Greater than 90% inhibition was produced by 3 μM when the psoas enzyme was tested and 50% inhibition required only about 0.5 μM. The liver enzyme was less sensitive, requiring 2 μM for 50% inhibition and 10 μM for 90%. There was very little difference between these values and those obtained when crude fractions from these tissues were used as a source of enzyme (Figure 4B). Inhibition by MGBG is less marked when mammalian *S*-adenosyl-

Table III: Inhibition of *S*-Adenosylmethionine Decarboxylase Activity from Various Tissues by MGBG and Congeners[a]

compound tested[b]	concn (μM) needed to achieve ~50% inhibition for tissue shown				
	liver	kidney	heart	psoas	diaphragm
methylglyoxal bis(guanylhydrazone)	2.0	2.0	1.8	0.5	0.5
ethylglyoxal bis(guanylhydrazone)	0.6	0.6	0.4	0.2	0.2
dimethylglyoxal bis(guanylhydrazone)	0.5	0.5	0.5	0.2	0.2
n-butylglyoxal bis(guanylhydrazone)	15	15	10	10	10
propanedialdehyde bis(guanylhydrazone)	3000	ND[c]	ND	2500	ND
pentanedialdehyde bis(guanylhydrazone)	2000	ND	ND	1000	ND

[a] Activity was assayed as in Figure 4B by using dialyzed ammonium sulfate precipitates from control (uninduced) rat tissues at a range of concentrations for each of the compounds shown. The concentration needed to produce 50% inhibition was then determined and is shown in the table. [b] Structural formulas for these compounds are given in Corti et al. (1974) and Dave et al. (1977). [c] Not determined.

methionine decarboxylases are assayed in the absence of putrescine (Williams-Ashman & Schenone, 1972; Corti et al., 1974). Therefore, we tested the effect of MGBG at pH 6.5 and 7.5 in phosphate buffer in the absence of putrescine. The liver enzyme required 5 μM for 50% inhibition whereas the psoas enzyme required only 2 μM (results not shown).

Many compounds related to MGBG have been synthesized, and some are known to be powerful inhibitors of mammalian *S*-adenosylmethionine decarboxylases (Corti et al., 1974; Dave et al., 1977). We, therefore, tested extracts from various tissues to see whether differences in the degree to which *S*-adenosylmethionine decarboxylase activity was inhibited existed for these drugs also. As shown in Table III and in agreement with the report of Corti et al. (1974) for rat prostate *S*-adenosylmethionine decarboxylase, the ethyl and dimethyl derivatives were more potent inhibitors than MGBG whereas the butyl derivative and related compounds derived from longer dialdehydes were less potent. Enzyme activity from kidney and heart resembled liver in sensitivity to MGBG and derivatives whereas the enzymes from diaphragm resembled that from psoas. The enzymes from liver and psoas differed in the degree of inhibition by all of the compounds tested, but none of the compounds showed a greater discrimination than MGBG itself.

Discussion

A number of possible artifacts which might have been responsible for the apparent differences observed by Pösö & Pegg (1981a,b) between rat liver and muscle *S*-adenosylmethionine decarboxylases can be eliminated on the basis of the present results. Contamination of one of the enzymes with the activity degrading *S*-adenosylmethionine and liberating CO_2 from the carboxyl carbon first reported by Sturman (1976a,b) could produce such an artifact in crude extracts, but this could not be the case with the present enzymes which gave single bands on gel electrophoresis under isoelectric focusing and denaturating conditions. The results with denaturing gels showing that enzymes from both sources have identical subunit M_r values also provide good evidence that the results are not due to proteolytic cleavage. This is also rendered unlikely by the high yield of homogeneous enzyme obtained from each tissue by the isolation procedure involving affinity chromatography. It is possible that the difference between the liver and psoas enzymes involves posttranslational modification although the quite wide difference in isoelectric point suggests a substantial alteration. Very large changes in isoelectric point can be produced by attachment of oligonucleotides to enzymes (Jonsson, 1981), but there was no difference between the extinction coefficient in the UV region for the purified muscle and liver enzymes, and the 280- to 260-nm absorbance ratio was 1.75, giving no indication of any attached nucleotide. Antisera against the rat liver enzyme prepared by immunization of rabbits (Pegg, 1979) were able to inhibit *S*-adenosylmethionine decarboxylase activity from a wide range of tissues including muscle (Pegg, 1979; Pegg et al., 1981), but further examination of this question by use of monospecific antibodies would be helpful.

S-Adenosylmethionine decarboxylase is present in very small amounts in control tissues, rendering it very difficult to obtain sufficient pure enzyme for characterization except after the 20–35-fold increase brought about by exposure to MGBG. Therefore, it cannot be certain that the uninduced forms of the enzyme correspond to those observed here, but there is considerable indirect evidence that this is the case. Crude preparations of uninduced enzyme show similar differences between psoas and liver to those seen with the highly purified enzymes in respect to activation by putrescine (Pösö & Pegg, 1981b) and to inhibition by MGBG (Table III). Furthermore, skeletal muscle *S*-adenosylmethionine decarboxylase is more sensitive to the negative regulatory influence exerted by spermidine than the liver enzyme (Pösö & Pegg, 1981b).

A convenient method for separation of the two forms of *S*-adenosylmethionine decarboxylase is chromatography on MGBG–Sepharose as in Figure 3. The explanation for the efficacy of this technique is clear from the results in Figure 4 showing that the muscle enzyme which is bound more tightly to MGBG–Sepharose is more sensitive to inhibition by MGBG and presumably has a greater affinity for the drug. It should be noted that the combination of greater sensitivity to MGBG and the higher MGBG concentration in fractions eluting the muscle enzyme render the dialysis of the fractions essential for detection of enzyme activity. In the original procedure developed in this laboratory for purification of *S*-adenosylmethionine decarboxylase by affinity chromatography on MGBG–Sepharose, the liver enzyme was purified, and the MGBG eluate could be assayed merely by diluting the fractions (Pegg, 1974, 1977). It is possible that the muscle form of the enzyme would be missed if this procedure is used without dialysis. In the present work, we checked all of the MGBG-containing eluate fractions after dialysis, and there was no sign of a later eluting form in the liver extracts or of the earlier form in the muscle extracts, but examination of other tissues might reveal multiple forms present in the same tissue.

The results obtained on inhibition of activity by MGBG (Figure 4) suggest that it may be possible to obtain a preliminary idea of the form(s) of *S*-adenosylmethionine decarboxylase present in a tissue by measuring the inhibiion by MGBG. There was essentially no difference between the results obtained with homogeneous enzyme and those obtained on assays of a crude, ammonium sulfate precipitated fraction. The ammonium sulfate fractionation may be essential for this comparison to be valid. It is known that sensitivity of *S*-adenosylmethionine decarboxylase to MGBG can be influenced by the presence of other molecules. For example, the mouse liver enzyme was an order of magnitude less sensitive than the rat liver *S*-adenosylmethionine decarboxylase when

fresh, centrifuged extracts were compared (Corti et al., 1974; Heby & Russell, 1973). However, a single-step purification consisting of precipitation with ammonium sulfate followed by dialysis removed the factors responsible for this difference and increased the sensitivity of the mouse liver enzyme (Corti et al., 1974).

The subsequent M_r of 32 500 found in the present work is in excellent agreement with our earlier estimates for rat liver *S*-adenosylmethionine decarboxylase (Pegg, 1974, 1977) and is somewhat smaller than the M_r of 42 000 reported by Demetriou et al. (1978). It also closely matches the M_r values of 32 000 reported for the mouse liver enzyme (Sakai et al., 1979) and for the bovine enzyme (Degen et al., 1981). Our estimate of the isoelectric point for the rat liver enzyme of 5.7 is also quite close to that of 5.9 for the mouse enzyme reported by Sakai et al. (1979). However, the activation of the mouse liver enzyme by putrescine was maximal with 5 μM diamine (Sakai et al., 1979) whereas the rat liver enzyme required 50–100 times this concentration (Table II). This difference could be related to the use of 0.1% deoxycholate to stabilize the pure enzyme since the crude enzyme from mouse liver not exposed to detergents also required much higher amounts of putrescine for full activity (Sakai et al., 1979). The finding that putrescine produces a 2–3-fold greater increase in the activity of the muscle enzyme compared to the liver form is in agreement with our previous results using crude tissue extracts (Pösö & Pegg, 1981b) except that the extent of stimulation is lower with the purified enzyme. The reason for this change is unclear, but it may be relevant that the rat liver and muscle enzymes are very unstable in the purified form when putrescine is omitted. A substantial proportion of the enzyme activity was lost during the dialysis to remove the putrescine prior to the measurements in Table II. This probably indicates that the enzyme is very readily denatured in the absence of putrescine and partial denaturation could contribute to the change in the degree to which activity is enhanced by putrescine.

The physiological relevance of the difference between liver and muscle *S*-adenosylmethionine decarboxylases could relate to the fact that muscle contains much lower concentrations of putrescine and spermidine than liver (McAnulty & Williams, 1977; Kremzner et al., 1978; Conover et al., 1980; Hopkins & Manchester, 1981; Pösö & Pegg, 1981b). The greater activation of *S*-adenosylmethionine decarboxylase by putrescine could provide a mechanism for maintaining a similar spermidine:putrescine ratio where actual putrescine levels are lower. Two other differences between the enzymes might relate to the much lower spermidine level in muscle compared to that in liver. First, the higher K_m for *S*-adenosylmethionine of the psoas enzyme and the lower content of this nucleoside in muscle (Eloranta, 1977) probably reduce the supply of decarboxylated *S*-adenosylmethionine needed for spermidine production. Second, *S*-adenosylmethionine decarboxylase is negatively regulated by spermidine (Hopkins & Manchester, 1980; Alhonen-Hongisto, 1980; Mamont et al., 1981; Mamont & Danzin, 1981; Pösö & Pegg, 1981b) possibly via a stimulatory effect of spermidine on the degradation of the enzyme (Mamont et al., 1981; Pösö & Pegg, 1981b). *S*-Adenosylmethionine decarboxylase in muscle is more sensitive to reduction by spermidine than is the liver enzyme (Pösö & Pegg, 1981b). This would tend to maintain muscle spermidine content at a lower value.

Irrespective of any physiological importance of the difference between *S*-adenosylmethionine decarboxylases from various tissues, the observations may have pharmacological relevance. Drugs affecting polyamine biosynthesis may have clinical value in treatment of neoplastic and parasitic diseases (Koch-Weser et al., 1981; McCann et al., 1981; Porter et al., 1981). MGBG has already been used as a cancer chemotherapeutic agent (Porter et al., 1981). The difference in sensitivity to MGBG and related compounds seen in Table III may give some degree of selectivity in the abilities of these drugs to inhibit the enzyme in various tissues, and the possible presence of different forms of the enzyme in tumors is of obvious interest. The present results also suggest that an attempt to synthesize even more selective inhibitors would be worthwhile.

References

Alhonen-Hongisto, L. (1980) *Biochem. J. 190*, 747–754.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Conover, C. A., Rozovski, S. J., Belur, E. R., Aoli, T. T., & Ruderman, N. B. (1980) *Biochem. J. 192*, 725–732.

Corti, A., Dave, C., Williams-Ashman, H. G., Mihich, E., & Schenone, A. (1974) *Biochem. J. 139*, 351–357.

Dave, C., Ehrke, M. J., & Mihich, E. (1977) *Chem.-Biol. Interact. 16*, 57–68.

Degen, J. L., Seyfried, C. E., & Morris, D. R. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1552.

Demetriou, A. A., Cohn, M. S., Tabor, C. W., & Tabor, H. (1978) *J. Biol. Chem. 253*, 1684–1686.

Eloranta, T. O. (1977) *Biochem. J. 166*, 521–529.

Eloranta, T. O., & Raina, A. (1978) *Biochem. Biophys. Res. Commun. 84*, 23–30.

Heby, O., & Russell, D. H. (1973) *Cancer Res. 33*, 159–165.

Hopkins, D., & Manchester, K. L. (1980) *FEBS Lett. 109*, 299–302.

Hopkins, D., & Manchester, K. L. (1981) *Biochem. J. 196*, 603–610.

Jänne, J., Pösö, H., & Raina, A. (1978) *Biochim. Biophys. Acta 473*, 241–293.

Jonsson, I. (1981) *Biochim. Biophys. Acta 660*, 204–213.

Koch-Weser, J., Schechter, P., Bey, P., Danzin, C., Fozard, J. R., Jung, J., Mamont, P. S., & Seiler, N. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 437–453, Marcel Dekker, New York.

Kremzner, L. T., Tennyson, U. M., & Miranda, A. F. (1978) *Adv. Polyamine Res. 2*, 241–256.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Mamont, P. S., & Danzin, C. (1981) *Adv. Polyamine Res. 3*, 123–135.

Mamont, P. S., Joder-Ohlenbusch, A. M., Nussli, M., & Grove, J. (1981) *Biochem. J. 196*, 603–610.

McAnulty, P. A., & Williams, J. P. G. (1977) *Biochem. J. 162*, 109–121.

McCann, P. P., Bacchi, C. J., Hanson, W. L., Cain, G. D., Nathan, H.-C., Hutner, S. H., & Sjoerdsma, A. (1981) *Adv. Polyamine Res. 3*, 97–110.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Pegg, A. E. (1974) *Biochem. J. 141*, 581–583.

Pegg, A. E. (1977) *FEBS Lett. 84*, 33–36.

Pegg, A. E. (1979) *J. Biol. Chem. 254*, 3249–3253.

Pegg, A. E., & Williams-Ashman, H. G. (1969) *J. Biol. Chem. 244*, 682–693.

Pegg, A. E., & Williams-Ashman, H. G. (1970) *Arch. Biochem. Biophys. 137*, 156–165.

Pegg, A. E., Hibasami, H., Matsui, I., & Bethell, D. R. (1981) *Adv. Enzyme Regul. 19*, 427–451.

Porter, C. W., Dave, C., & Mihich, E. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 407–436, Marcel Dekker, New York.

Pösö, H., & Pegg, A. E. (1981a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1683.
Pösö, H., & Pegg, A. E. (1981b) *Biochem. J. 200*, 629-637.
Sakai, T., Hori, C., Kano, K., & Oka, T. (1979) *Biochemistry 18*, 5541-5548.
Sturman, J. A. (1976a) *Biochim. Biophys. Acta 428*, 56-69.
Sturman, J. A. (1976b) *Life Sci. 18*, 879-886.
Tabor, C. W., & Tabor, H. (1976) *Annu. Rev. Biochem. 45*, 285-306.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384-421.
Williams-Ashman, H. G., & Schenone, A. (1972) *Biochem. Biophys. Res. Commun. 46*, 288-295.
Williams-Ashman, H. G., & Pegg, A. E. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 43-73, Marcel Dekker, New York.
Wilson, J., Corti, A., Hawkins, M., Williams-Ashman, H. G., & Pegg, A. E. (1979) *Biochem. J. 180*, 515-522.

Laser Flash Photolysis Studies of Electron Transfer between Semiquinone and Fully Reduced Free Flavins and the Cytochrome *c*-Cytochrome Oxidase Complex†

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ABSTRACT: Laser flash photolysis has been used to determine the rate constants for the reduction of bovine cytochrome oxidase and the cytochrome *c*-cytochrome oxidase complex by the semiquinone and fully reduced forms of various flavin analogues ($FH^{\cdot}$ and FH^{-}, respectively). Under the conditions used, the reaction of $FH^{\cdot}$ with free cytochrome oxidase is too slow to compete with $FH^{\cdot}$ disproportionation whereas FH^{-} reacts measurably. Both $FH^{\cdot}$ and FH^{-} are effective in reducing the complex. The reduction of heme *a* in the complex is shown to proceed via cytochrome *c*, and a limiting first-order rate is observed in the case of FH^{-} at high complex concentrations. The data indicate that the interaction site for electron transfer to cytochrome *c* is the same in the complex as with the free protein, and although a tight complex exists, at least small reactants like the flavins are not sterically hindered in their access to the bound cytochrome *c*. Moreover, the results also establish that intramolecular electron transfer between cytochrome *c* and cytochrome oxidase within the complex occurs with a first-order rate constant of greater than 700 s^{-1}. Thus, the presence of cytochrome *c* greatly enhances electron transfer from reduced flavins to cytochrome oxidase.

Although cyt oxidase[1] has been intensively investigated, many aspects of its molecular mechanism remain to be elucidated [cf., for example, Malmström (1979)]. This results in part from a lack of structural information. A large number of studies have also been carried out on the interaction of cyt *c* with cyt oxidase, but in spite of the extensive structural information available concerning cyt *c*, the details of the interaction are still not well understood (Malmström, 1979; Hill & Nicholls, 1980). This results in part from limitations inherent in the methodology used. Thus, the reaction rate is at the borderline of the stopped-flow technique ($k \sim 4 \times 10^7$ M^{-1} s^{-1}), and the interpretation of steady-state studies is difficult inasmuch as a mechanism must be assumed and in general individual rate constants are not easily resolved.

Recently, we have applied a rather different kinetic approach to the study of biological oxidation-reduction reactions (Cusanovich & Tollin, 1980; Ahmad et al., 1981). Since free flavins can be photochemically reduced with a short pulse of laser light, a direct means of producing a strong reducing agent in a short time period (<1 μs) is available. Moreover, substantial information has been accumulated concerning the physical-chemical and kinetic properties of free flavins, as well as a wide variety of flavin analogues. We have used this approach to study intramolecular electron transfer in *Chromatium vinosum* cytochrome *c*-522, a complex cytochrome containing two hemes and a flavin moiety, and to derive information about the electrostatics, steric restrictions, and nonpolar interactions at the site of electron transfer between ferricyt *c* and fully reduced and semiquinone flavins. We report here on a similar study of the reduction of cyt oxidase and the cyt *c*-cyt oxidase complex with a variety of photochemically reduced flavins.

Materials and Methods

Riboflavin, FMN (sodium salt), and cytochrome *c* (horse heart, ferri form, type VI) were obtained from Sigma Chemical Co. Lumiflavin, 10-methylisoalloxazine, and 7,8-dichlororiboflavin were synthesized as previously described (Guzzo & Tollin, 1963; Shiga & Tollin, 1976). 8α-[*S*-(*N*-Acetyl)-L-cysteinyl]tetraacetylriboflavin was a gift from Dr. D. B. McCormick. The structures of the flavin derivatives used in this work are shown in Figure 1. Cytochrome oxidase was prepared from beef hearts as described previously (Yonetani, 1960). All other materials were analytical grade or of the purest form available from Fisher Scientific Co. and Mallinckrodt Chemical Co.

Solutions for flash photolysis, which contained 0.1 mM flavin, 5 mM EDTA, and suitable concentrations of cyt oxidase (concentration calculated on the basis of two hemes per

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[1] LF, lumiflavin; RF, riboflavin; FMN, flavin mononucleotide; 10-MI, 10-methylisoalloxazine; Cl_2RF, 7,8-dichlororiboflavin; Ac-Cys-TARF, 8α-[*S*-(*N*-acetyl)-L-cysteinyl]tetraacetylriboflavin; $FH^{\cdot}$, neutral flavin semiquinone; FH^{-}, fully reduced flavin; cyt *c*, horse heart cytochrome *c*; cyt oxidase, beef heart cytochrome oxidase; EDTA, ethylenediaminetetraacetic acid.

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