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KINETIC PROPERTIES OF *S*-ADENOSYLMETHIONINE:Δ^{24}-STEROL METHYLTRANSFERASE ENZYME(S) IN MITOCHONDRIAL STRUCTURES OF *SACCHAROMYCES CEREVISIAE*

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SUMMARY

The inhibition of *S*-adenosylmethionine:Δ^{24}-sterol methyltransferase (EC 2.1.1.41) activity by endogenous cellular components has been studied in vitro. The principal inhibitors were Na^+ and K^+; Cs^+, NH_4^+ and Li^+ were also shown to inhibit the reaction. The possible significance of inhibition by sodium and potassium is discussed.

Evidence is presented for the presence of more than one enzyme capable of methylating sterols in cell-free extracts of yeast. Three enzymatic activities are described which differ in their respective Michaelis constants for *S*-adenosyl-L-methionine, pH optima, and affinity for zymosterol. Based on differences in the apparent Michaelis constants for zymosterol, it appears that only one may be responsible for in vivo methylation of this ergosterol precursor.

INTRODUCTION

The principal biosynthetic pathway for ergosterol in yeast is still unknown, but it has been established that C-28 is derived from a transmethylation via *S*-adenosyl-L-methionine [1] while the rest of the molecule is synthesized from acetate. Ergosterol is the major sterolic component of yeast, comprising up to 10% of the dry weight of aerobically grown yeast [2]. Recently, however, several C-24-methylated intermediates have been isolated and identified [3, 4]. Zymosterol is believed to be the substrate for methylation [5], although one group has reported it to inhibit crude cell-free enzyme preparations [6].

Extensive research has been done recently on an enzyme catalyzing this transmethylation, *S*-adeonosylmethionine: Δ^{24}-sterol methyltransferase (EC 2.1.1.41). Turner and Parks [7] have shown that Cu^{2+} will almost totally inhibit cell-free enzyme preprations, while Ca^{2+} was reported to give slight inhibition. The same researchers found a carbonate requirement for maximal activity. Moore and Gaylor [8] have reported both magnesium and glutathione requirements for an acetone powder preparation of the methyltransferase enzyme. The subcellular localization of

transmethylation activity has been shown to reside in yeast mitochondria and promitochondria (preceding paper).

An accumulation of indirect evidence [5, 6, 8] suggests that the methyltransferase enzyme may be a control point in ergosterol biosynthesis. Ergosterol inhibits the methylation of zymosterol which implies a feed-back regulation mechanism [8]. This communication presents evidence for the existence of more than one methyltransferase enzyme in yeast mitochondrial and promitochondrial structures. Inhibitory effects of monovalent cations normally present in cell-free enzyme preparations of yeast also have been examined.

EXPERIMENTAL PROCEDURES

Organism and cultural conditions

Saccharomyces cerevisiae, strain 3701-B, a haploid organism with a uracil requirement was used throughout this study. The organism was maintained on agar slants containing 1% tryptone, 0.5% yeast extract, and 2% glucose. The yeast was grown in a similar broth in which 2% peptone replaced the tryptone. A 10% (v/v) inoculum was added to 500 ml of this medium in a 2-l flask and incubated overnight at 30 °C in a New Brunswick rotary shaker. The cells were harvested by centrifugation at 1000 × *g*. The final yield was 20–25 g wet weight of cells/l.

Enzyme preparation

The harvested cells were washed twice with 0.1 M Tris–HCl buffer, pH 7.5, and resuspended in the same buffer to 1 g cells/ml. All subsequent steps were carried out at 4 °C. The cells were broken in a Bronwill MSK tissue homogenizer as described in the preceding paper. The homogenate was centrifuged at 25 000 × *g* for 20 min and the pellet discarded. The supernatant was recentrifuged for 1 h at 105 000 × *g* in a Spinco model L2-65 preparative ultracentrifuge. The floating lipid layer was removed with a Pasteur pipette. The supernatant was decanted and recentrifuged at 105 000 × *g* to remove any slowly settling particles. The clear supernatant from this centrifugation (designated S_{105}) was then stored at −20 °C until use.

The sediment from the first 105 000 × *g* centrifugation containing the active sterol methyltransferase enzyme was resuspended to 15 mg protein/ml in 0.1 M Tris–HCl buffer, pH 7.5. The protein concentration was determined by the method of Lowry et al. [8]. This suspension was then stored at −20 °C until needed. The enzyme is stable at this temperature for at least three weeks.

Mitochondria were isolated as reported in the preceding paper.

Transmethylation assay procedure

The enzyme assays were performed as reported in the preceding paper with the following modifications. The amount of *S*-adenosylmethionine added varied from 2.5 nmoles (0.58 μM) to 400 nmoles (93 μM) depending on the K_m level under observation. Appropriate dilutions of the labelled material were made with carrier *S*-adenosylmethionine. The reaction volume was kept constant at 4.3 ml throughout these studies by varying the amount of buffer.

Measurement of S-adenosylmethionine

Freshly prepared 0.4 cm × 3 cm Dowex 50W-X8 (50–100 mesh) columns charged with LiCl were employed to determine *S*-adenosylmethionine levels before and after incubation with the S_{105} fraction. 1.25 nmoles of *S*-adenosyl[^{14}C]methionine were added to 1.0 ml of the S_{105} and the mixture incubated for 30 min at 30 °C. Portions (0.2 ml) removed at zero time and after 30 min incubation were applied to the Dowex columns. The columns were washed with 24 ml of distilled water after which the *S*-adenosyl[^{14}C]methionine was eluted with 15 ml of 3 M NH_4OH. 1-ml samples from the water wash and the NH_4OH eluate were counted separately in 10 ml of Bray's solution [9] on a Packard Tri-Carb liquid scintillation counter. Essentially all of the *S*-adenosyl[^{14}C]methionine added to the column was recoverable in the NH_4OH fraction.

Atomic absorption studies on S_{105}

A Perkin–Elmer 290B atomic absorption spectrophotometer was used to detect the presence of cations in the S_{105}. The presence of a particular cation was taken as a positive difference between the absorption value for the S_{105} and that obtained for the Tris–HCl buffer.

pH curves

Buffers were prepared ranging from pH 5.5 to 8.5 and increasing by increments of 0.5 pH unit. All buffers were 0.1 M. At pH values of 5.5, 6.0, 6.5 and 7.0, phosphate buffers were used, while a Tris–HCl buffer was employed at higher pH values. Overlapping of the two buffers used at pH 6.5 and 7.0 was employed to correct enzyme activities to those obtained in Tris–HCl buffer only. Enzyme assays were then performed using the different buffers as described.

RESULTS

Early studies showed that portions of the 105 000 × *g* supernatant (S_{105}) added to the cell-free reaction produced maximally an 80% reduction in methyltransferase activity. Fig. 1 depicts the type of inhibition achieved with addition of both 1.0 and 2.0 ml of S_{105} to the assay system. This 80% inhibition occurred with complete substitution of the buffer by S_{105} (3.0 ml). The degree of inhibition by a given amount (generally 1.0 ml) of S_{105} varied from preparation to preparation. 1 ml of S_{105} inhibited the standard methyltransferase assay from 50 to 75%.

The possible presence of an *S*-adenosylmethionine cleaving enzyme [12] in the S_{105} was investigated by allowing incubation of the *S*-adenosylmethionine with S_{105} for 30 min. Samples taken at the beginning and end of the 30-min incubation period showed virtually no change in the *S*-adenosylmethionine level, thus eliminating the possibility of an *S*-adenosylmethionine cleaving enzyme. The possibility of non-sterolic methyltransferase substrates present in the S_{105} (i.e. tRNA, RNA, DNA) combined with a non-specific methyltransferase in the enzyme preparation was also investigated. The presence of such a methyltransferase would cause competitive inhibition. Fig. 1 rules out this possibility since addition of the S_{105} results in typical non-competitive inhibition.

Attempts were then made to characterize and identify the methyltransferase

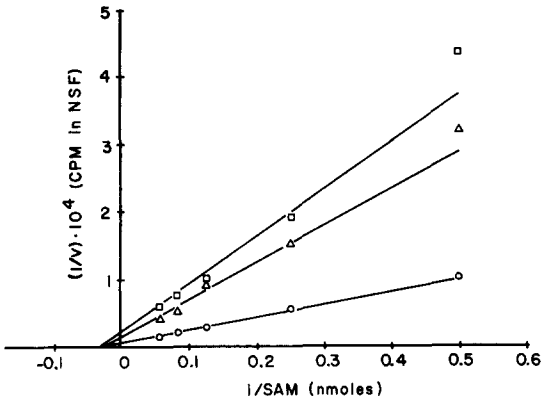

Fig. 1. Non-competitive inhibition of the methyltransferase enzyme inflicted by S_{105}. The assay procedure is detailed in Experimental Procedures. The transmethylation reactions were performed with varying *S*-adenosylmethionine (SAM) concentrations. Enzyme velocity is expressed as cpm incorporated into the non-saponifiable fraction (NSF). Reciprocal plots have been made of the transmethylation rates obtained with the enzyme alone (○—○), 1.0 ml of added S_{105} (△—△), and 2.0 ml of added S_{105} (□—□).

inhibitor present in the S_{105} fraction. Table I shows the inhibitor to be completely removed by dialysis and to be heat stable to a temperature of 100 °C for 1 min. Prolonged heating of the S_{105} at 100 °C still did not inactivate the inhibitor. To investigate further the nature of the inhibitory component, several 1.0-ml volumes of S_{105} were ashed at 600 °C for 2 h. The ashing was performed in the same culture tubes used for the transmethylase assay to prevent unnecessary loss of the ash. After resuspending the resultant ash in 1.0 ml of Tris–HCl buffer, the enzyme assay reagents were added to the tube. Table I indicates that ashing removes only 30% of the inhibition. The majority of the inhibitory material thus appeared to be inorganic as evidenced by its stability to ashing. Atomic absorption spectrometry was used to determine the nature of the cations in the S_{105}. Known inhibitors of the methyltransferase enzyme such as copper and calcium [6] were not present. However, sodium

TABLE I

EVIDENCE FOR INORGANIC NATURE OF METHYLTRANSFERASE INHIBITOR PRESENT IN S_{105}

Various physical treatments of the S_{105} fraction are shown with the resulting inhibition obtained when 1.0 ml of the treated S_{105} is added to the methyltransferase assay as described. Values shown are representative of three separate experiments.

Treatment of S_{105}	cpm in non-saponifiable lipids	Inhibition (%)
Control (minus S_{105})	23 100	–
Control (plus 1.0 ml S_{105})	7 600	68
24-h dialysis (against 200 vol. of 0.1 M Tris–HCl, pH 7.5)	23 600	0
Boiling (100 °C for 1 min)	6 420	72
Ashing (600 °C for 2 h)	11 600	50

and potassium were found in the S_{105} in large amounts. K^+ was found in quantities exceeding 30 mM, while Na^+ was present at levels of 9–10 mM.

The possible inhibition of the methyltransferase by these and other monovalent cations was then examined. Table II lists the cations tested showing the degree each inhibits the methyltransferase enzyme. In addition to the chloride salts used in Table II, several other salts of each cation were tested. There was virtually no variance in the inhibition achieved by these different salts. Sodium was by far the most effective

TABLE II

INHIBITION OF STEROL METHYLTRANSFERASE BY VARIOUS MONOVALENT CATIONS

Values reported are averages of two different experiments. Each salt is present in the enzyme assay at a concentration of 25 mM. In each case the percent inhibition is maximal for the particular cation.

Cation tested	Inhibition (%)
Na^+	65
K^+	31
NH_4^+	35
Cs^+	27
Li^+	32

inhibitor of the transmethylation reaction. A hyperbolic relationship between NaCl concentration and enzyme inhibition was observed with a maximum of 65% inhibition occurring in the presence of 25 mM NaCl. The inhibition by different monovalent cations was found to be additive and when the methyltransferase assay was performed under conditions identical to those obtained by adding aliquots of S_{105} 45–50% maximal inhibition was observed.

Since 30% of the inhibition was removed by ashing, an analysis of the S_{105} for inhibitory organic compounds was made. The presence of ergosterol, a known methyltransferase inhibitor [8], was detected in saponified portions of S_{105}. However, association of the ergosterol with small dialysable cellular components was suspected since the S_{105} was refractory to hexane extraction without prior saponification. Glycine was also tested for inhibitory action [13] in our assay system with negative results.

Variation of the *S*-adenosylmethionine concentration produced atypical Michaelis–Menten kinetics as shown in Fig. 2. The two plateau regions of the curve always appeared at the same relative substrate concentrations, and three different slopes were consistantly observed between these plateaus. The insert represents the velocity obtained at very low substrate concentrations and clearly shows a plateau between 1 and 1.5 μM *S*-adenosylmethionine. The second plateau exists between substrate concentrations of 27 and 30 μM. The same overall pattern also was observed when only endogenous sterolic substrates were employed.

The sterolic products from the methyltransferase reaction remain the same at the different *S*-adenosylmethionine concentrations as measured by migration on thin-layer chromatography with Silica gel G (benzene–ethyl acetate, 5:1, v/v). Only sterols which migrated at R_F values corresponding to fecosterol and ergosterol [3] (R_F = 0.33 and 0.13, respectively) incorporated the labelled methyl group in all three cases. It was noticed, however, that the proportion of [^{14}C]ergosterol increased from

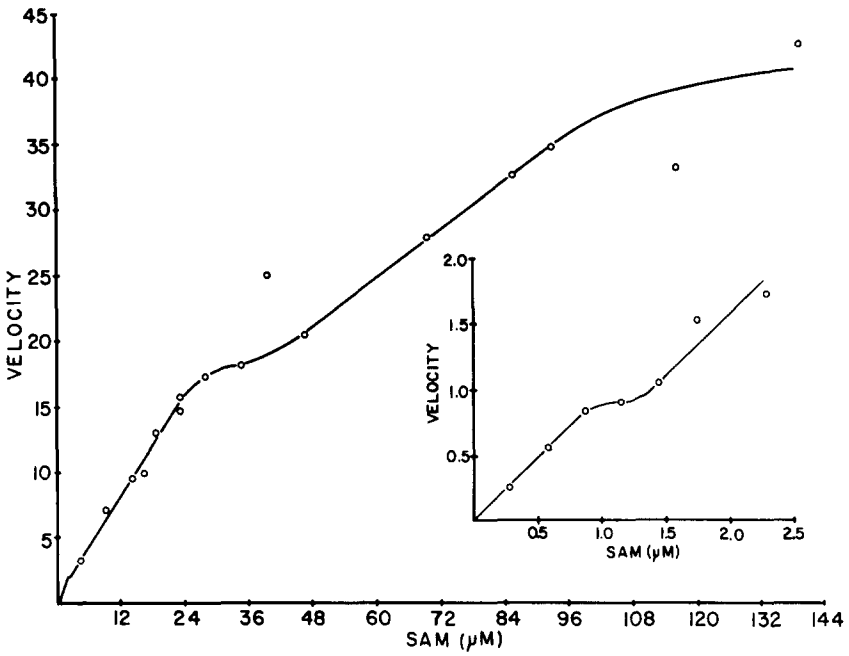

Fig. 2. Michaelis–Menten plot of enzyme velocity versus *S*-adenosylmethionine (SAM) concentration. Velocity is expressed as nmoles product formed/h per 15 mg protein. The insert represents the lower substrate concentrations which cannot be detailed on the larger scale.

8 to 45% of the total labelled non-saponifiable lipids with increasing *S*-adenosylmethionine concentrations.

Fig. 3 (A, B, and C) shows standard Lineweaver–Burk plots [11] of the three different slopes observed on the Michaelis–Menten curve (Fig. 2). The average (three experiments) apparent Michaelis constants (K_m) for *S*-adenosylmethionine are 4, 32, and 110 μM, respectively. Fig. 3 also shows all three phases to be inhibited by Na^+ in a non-competitive fashion. The degree of inhibition of the methyltransferase reaction was independent of the substrate concentration and maximal inhibition was 65–70%.

The methyltransferase enzyme preparation was assayed in buffers ranging from pH 5.5 to 8.5 as detailed in Experimental Procedures. The results are represented by Fig. 4. The highest enzymatic activity found in a given series of reactions was assigned a value of 100%. Percent activity as shown was thus calculated as a fraction of the "100% activity" assay. The optimal pH consistantly varied from pH 7.0 to 7.7 with increasing *S*-adenosylmethionine concentrations. The pH of the reaction mixture did not change during the incubation period.

Since the preceding data indicated the presence of more than one enzyme capable of methylating zymosterol, the exogenously supplied zymosterol concentration was varied to obtain K_m values for this substrate. At both the low (0.58 μM) and intermediate (10 μM) *S*-adenosylmethionine levels, an apparent K_m of 100 μM for zymosterol was obtained. However, at a substrate concentration of 93 μM, a K_m (zymosterol) of 55 μM was found.

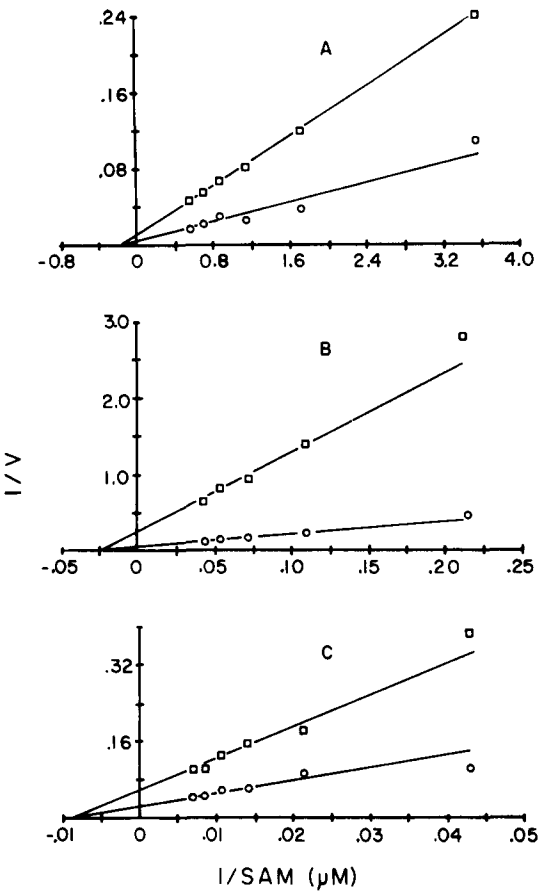

Fig. 3. Lineweaver–Burk plots of the three slopes obtained on the Michaelis–Menten plot (Fig. 2). Enzyme assay procedure is detailed in Experimental Procedures. The *S*-adenosylmethionine (SAM) concentration was varied from 0.29 to 1.5 μM in A; from 1.5 to 30 μM in B, and from 30 to 140 μM in C. Velocity is shown as cpm/h per 15 mg protein. The apparent K_m values obtained for this set of experiments were 3.5 μM for A, 38 μM for B, and 110 μM for C. (○—○), transmethylation rates with enzyme alone; (□—□), transmethylation rates in the presence of 25 mM NaCl.

The digitonin method of mitochondrial fractionation [16] was used to attempt the localization of the three enzymes in yeast mitochondria. All three enzymatic activities were found in the inner-membrane and matrix portions of the mitochondria (preceding paper) with the majority of all three being found in the matrix. A Michaelis–Menten plot exactly like Fig. 2 was obtained when the matrix fraction was used as the enzyme source confirming the presence of the three enzymes.

DISCUSSION

Several investigators [5–8] have examined the sterol methyltransferase process in yeast with slightly conflicting results. While zymosterol has been shown to be the most efficiently used substrate [5, 8], one group [6] has reported that exogenously

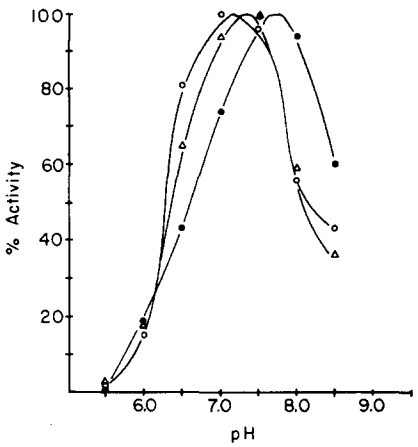

Fig. 4. pH curves for the three methyltransferase activities. Both 0.1 M Tris–HCl buffer and 0.1 M phosphate buffer were used in these experiments. (○—○), 0.58 μM *S*-adenosylmethionine; (△—△), 10 μM *S*-adenosylmethionine; (○—○), 93 μM *S*-adenosylmethionine. pH optima were 7.1, 7.3, and 7.7, respectively.

added zymosterol inhibited their cell-free methyltransferase enzyme preparation. Conflicting and varied co-factor requirements also have been reported [5, 7].

Addition of zymosterol to our cell-free enzyme preparation did not inhibit the methyltransferase activity as previously reported [6]. To the contrary, added zymosterol greatly stimulated the reaction (6-fold). However, we found that zymosterol spontaneously breaks down as shown by the appearance of two distinct melting points. This resulted in a presumably unsuitable substrate which did inhibit the enzyme. Instead of the expected stimulation by the substrate, there was a considerable amount of inhibition imparted by the degraded zymosterol. The nature of the degradation is not known. For this reason, it was necessary to make up zymosterol in absolute ethanol on the day it was to be used. It is relatively stable when stored at −15 °C as a powder.

The magnesium and carbonate requirements [7] were not absolute, but both were necessary for full enzymatic activity. Unlike Moore and Gaylor [5] we found no glutathione requirement in our system.

The non-competitive inhibition of the methyltransferase enzyme(s) in vitro by the S_{105} fraction seems predominately due to the presence of a mixture of monovalent cations. The S_{105} inhibitor(s) is completely dialysable ,heat stable, and partially stable to ashing at 600 °C for 2 h. The 25–30% loss of inhibition consistantly observed after ashing indicates that some small molecular weight organic component of the S_{105} fraction was also inhibiting the enzyme. However, the high concentrations of both K^+ (30–35 μM) and Na^+ (9–10 μM) found in the S_{105} are sufficient to inhibit the standard enzyme assay by 45–50% maximally. A "synthetic S_{105}" made up in Tris–HCl buffer to the above cation concentrations did in fact give 45–50% inhibition of the reaction. Thus 60–65% of the inhibition inflicted by the S_{105} is apparently due to these cations.

The remainder of the inhibition was attributed to the presence of ergosterol, a known methyltransferase inhibitor [8], in the S_{105}. Though Fig. 1 clearly depicts

non-competitive inhibition inflicted on the enzyme by S_{105} addition, the *S*-adenosylmethionine concentration was varied in these experiments and thus the competitive inhibition by ergosterol as previously reported [8] would not be observed. Kerr's recent publication [13] showing glycine to inhibit tRNA methyltransferases, led us to look for this effect in our system. Accordingly, glycine was tested for inhibitory action with negative results.

The variation in inhibition by a given amount of different S_{105} preparations probably reflects the degree of cell breakage since the cations were presumably released during homogenization. Also the resuspension to 1 g cells/ml prior to homogenization was dependent upon wet weight estimations and thus subject to some variance. Thus the S_{105} was not standardized as far as cation or protein content from preparation to preparation.

Since the three methyltransferase activities in yeast have been shown to reside in the mitochondria (data not shown), it is tempting to consider the sodium and potassium caused inhibition as a means of regulating sterol biosynthesis. Studies on ion transport induction have led to the suggestion that cation transport through the inner mitochondrial membrane is an energy-linked process [14, 15]. Because of this, it is feasible to assume that under conditions of active sodium or potassium transport and accumulation in mitochondria, sterol methyltransferase activity (and possibly other energy requiring processes) would be decelerated.

The kinetic pattern obtained (Fig. 2) by varying the *S*-adenosylmethionine concentration has several interesting ramifications. Similar patterns also were observed when only endogerous sterol substrate was used. This seems to eliminate the possibility of endogenous substrates being methylated preferentially at different velocities than the exogenously supplied zymosterol.

The increased accumulation of [^{14}C]ergosterol in the non-saponifiable fraction at higher *S*-adenosylmethionine concentrations is very interesting. This was attributed to enhanced fecosterol accumulation at maximal *S*-adenosylmethionine levels stimulating a more rapid conversion to ergosterol. Further speculation on these observations must await individual purification and characterization of these enzymes.

The kinetic data for both substrates are summarized in Table III. It appears that there may be three different enzymes present in cell-free yeast extracts capable of methylating zymosterol. The enzymes differ on the basis of their affinity for *S*-adenosylmethionine, pH optima, and apparent Michaelis constants for zymosterol. It should be pointed out that the K_m values derived for zymosterol were calculated in the presence of endogenous substrate. However, this was controlled by subtracting the velocity obtained in the absence of exogenously added zymosterol from the observed activity. Otherwise, all assays were treated identically. *S*-Adenosylmethionine concentrations used to determine zymosterol K_m values were those calculated to give a minimal contribution to the velocity from the other enzyme activities.

The lower zymosterol K_m for the third enzyme implies that it is more specific toward zymosterol as a substrate than the other enzymes. Both the pH optimum of 7.7 and the K_m (zymosterol) of 55 μM agree well with previously reported values. Moore and Gaylor [5, 8] found a pH optimum of 7.5 and a zymosterol K_m of 62.5 μM using similar substrate levels in an acetone powder preparation.

The presence of three enzymes capable of methylating zymosterol in the inner membrane and matrix portions of yeast mitochondria was somewhat unexpected.

TABLE III

SUMMARY OF KINETIC DATA FOR *S*-ADENOSYLMETHIONINE: Δ^{24}-STEROL METHYLTRANSFERASE ACTIVITIES

Enzyme activities were arbitrarily designated 1, 2, and 3 for reference purposes. V_{total} was calculated by extrapolation to infinite substrate concentration from Lineweaver–Burk plots (Fig. 4). Each individual V was then determined by subtracting the V of the preceding enzyme(s) (next lower K_m values) from the V_{total} value.

Methyltransferase activity	pH optimum	K_m zymosterol (μM)	K_m *S*-adenosylmethionine (μM)	V (nmoles product/h per 15 mg)
1	7.1	100	4	5
2	7.3	100	32	26
3	7.7	55	110	49

The significance of having three enzymes catalyzing the same reaction in the same subcellular location is unclear. Possibly only one of these three (K_m (zymosterol) of 55 μM) is specific for zymosterol methylation in vitro. The presence of a "transmethylation complex" of three enzymes in the mitochondria, responsible for methylating different sterol substrates, cannot yet be eliminated. Experiments are currently in progress to separate and characterize the three methyltransferase activities in order to clarify this problem.

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