Source of Methylmalonyl-Coenzyme A for Erythromycin Synthesis: Methylmalonyl-Coenzyme A Mutase from *Streptomyces erythreus*[†]

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Streptomyces erythreus produces erythromycin, presumably from methylmalonyl-coenzyme A (CoA), which could be generated by the isomerization of succinyl-CoA. In *S. erythreus* cultures, [1,4-¹⁴C,2,3-³H]succinate was incorporated into erythromycin with a doubling of the ³H/¹⁴C ratio. This result is consistent with the hypothesis that succinyl-CoA is isomerized to methylmalonyl-CoA before incorporation into the macrocyclic lactone of erythromycin. The presence of methylmalonyl-CoA mutase, which catalyzes this isomerization, was demonstrated in cell-free extracts prepared from this organism. Consistent with the suggested role for this enzyme, methylmalonyl-CoA mutase activity increased over 12-fold at the time of the most rapid antibiotic production, and the activity level drastically declined when the antibiotic production ceased. The mutase was partially purified from this organism with DEAE-cellulose, ammonium sulfate precipitation, and affinity chromatography on a B₁₂-coenzyme Sepharose column. The enzyme was stimulated 2.5-fold by the addition of B₁₂-coenzyme. The enzyme showed a typical Michaelis-Menten type substrate saturation patterns, with *K_m*s of 0.31 mM and 0.09 μM for methylmalonyl-CoA and B₁₂-coenzyme, respectively, and a *V* of 0.5 μmol/min per mg. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed a major band with a molecular weight of 63,000. The properties of this enzyme appear to be fairly similar to those of the mutase previously obtained from other sources.

The aglycone portion of erythromycin produced by *Streptomyces erythreus* is most probably synthesized from one molecule of propionyl-coenzyme A (CoA) and six molecules of methylmalonyl-CoA (1). Although it is clear that propionate and methylmalonate are precursors of erythromycin, how these precursors are generated is still unclear (1). Methylmalonyl-CoA could be generated either from carboxylation of propionyl-CoA by a carboxylase or by isomerization of succinyl-CoA by methylmalonyl-CoA mutase. A carboxylase which was recently purified from *S. erythreus* was found to use propionyl-CoA as the preferred substrate (6). Its role in the biosynthesis of erythromycin, however, is not well established. Since carbohydrates support antibiotic production by furnishing the carbon atoms of erythromycin (3), succinyl-CoA generated from the carbohydrate might be a major source for methylmalonyl-CoA. Early isotope studies (18) have suggested that acetate and pyruvate are incorporated into erythromycin via isomerization of succinyl-CoA to methylmalonyl-CoA, and direct incorporation of succinate into erythromycin has also been observed (5). Recent studies with ¹³C-labeled nuclear magnetic resonance analysis showed that succinate is probably the source of methylmalonate used in rifamycin (11) and tylosin (14) syntheses. However, methylmalonyl-CoA mutase, the enzyme involved in the isomerization, has not been studied from any antibiotic-producing organism. In this paper, we present dual isotope incorporation studies, the results of which suggest that exogenous succinate is incorporated into erythromycin after its isomerization to methylmalonate. We also demonstrate the occurrence of methylmalonyl-CoA mutase in the cell-free preparation from *S. erythreus* and report partial purification and characterization of this enzyme.

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MATERIALS AND METHODS

Materials. [1,4-¹⁴C]succinate (60 Ci/mol), [1,4-¹⁴C]succinic anhydride (118 Ci/mol), and [methyl-³H]methylmalonic acid (56 Ci/mol) were purchased from New England Nuclear Corp., Boston, Mass., and [2,3-³H]succinate (25 Ci/mol) was obtained from Rose Chemicals, Los Angeles, Calif. [1,4-¹⁴C]succinyl-CoA was prepared from [1,4-¹⁴C]succinic anhydride (16), and [methyl-³H]methylmalonyl-CoA was prepared from [methyl-³H]methylmalonic acid (15). The products were purified by high-pressure liquid chromatography as described previously (2). All other chemicals and materials were purchased from Sigma Chemical Co., St. Louis, Mo. The B₁₂-coenzyme Sepharose affinity gel was prepared by a method previously described (9).

Culture conditions. *S. erythreus* CA₃₄₀, obtained from Abbott Laboratories, North Chicago, Ill., was maintained as previously described (6). The slant cultures were used to inoculate the vegetative growth medium consisting of the following components (in the indicated amounts per liter), and the pH was adjusted to 7.0 with 10% KOH: sucrose (15.0 g), peptone (Difco; 5.0 g), yeast extract (2.5 g), L-arginine (0.5 g), and EDTA (35.0 mg). A 50-ml culture was grown in the vegetative medium in a 125-ml flask at 32°C in a rotary shaker operating at 250 rpm for 3 days. The resulting vegetative inoculum was then completely transferred into 1 liter of a medium containing 15 g of sucrose and 2 g of L-alanine in 2,800-ml Fernbach flasks and incubated at 32°C on a rotary shaker (250 rpm) for 48 h. Usually, six flasks were set up, the cells from one flask were harvested at desired intervals for time-course studies, and the enzyme level was measured.

Preparation of crude extract. Cells were harvested by centrifugation at 12,000 × *g* for 15 min, washed twice with 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM dithioerythritol (DTE), and resuspended in an equal volume of the same buffer. All further steps were carried out at 0 to

4°C. The cells were ruptured with a French pressure cell operating at 14,000 lb/in² (passed twice), the extract was centrifuged at 37,000 × *g* for 30 min, and the supernatant was the source of the enzyme.

In vivo succinate incorporation. A 5-ml inoculum from the vegetative culture was transferred to a 250-ml Erlenmeyer flask containing 50 ml of a medium consisting of 1.5% sucrose, 0.2% L-alanine, and tap water. The flasks were shaken at 32°C for 24 h. Approximately 25 µCi of [1,4-¹⁴C]succinate and 25 µCi of [2,3-³H]succinate were mixed to give a ³H/¹⁴C ratio of 0.9, and the mixture was added to the medium. After 12 h of incubation of the cultures with the radioactive substrate, the cells were removed by centrifugation, the supernatant was adjusted to pH 9.8 by the addition of 10% NaOH, and erythromycin was extracted with three volumes of ethyl acetate. The extract was concentrated to dryness, and the products were analyzed by thin-layer chromatography (silica gel) with ethyl acetate–2-propanol–15% aqueous (wt/vol) ammonium acetate adjusted to pH 9.8 (9:7:8, vol/vol/vol). Erythromycin and erythronolide B (Abbott Laboratories) were used as standards, and the radioactivity in the erythromycin zone was eluted from the plate with ethyl acetate. A sample of the eluted material was used to determine the radioactivity, and the rest was concentrated to dryness and used for high-pressure liquid chromatographic analysis as described previously (17). A µBondapak C₁₈ reversed-phase column was utilized with acetonitrile–methanol–0.2 M ammonium acetate–water (9:2:2:7, vol/vol/vol/vol) as the mobile phase. The radioactive erythromycin fraction obtained by thin-layer chromatography was dissolved in the above solvent system and subjected to high-pressure liquid chromatography, and the radioactivity in each fraction of the eluant (1 ml per fraction) was measured.

Product identification. The cell-free extract (0.76 mg) was incubated in the dark at 30°C with 4.5 µmol of [1,4-¹⁴C]succinyl-CoA (specific activity, 20 Ci/mol) and 60 µmol of B₁₂-coenzyme in a total volume of 200 µl. After 15 min of incubation, 20 µl of perchloric acid was added, and the precipitated proteins were removed by centrifugation. The products of the reaction were analyzed by high-pressure liquid chromatography as described previously (2).

Purification of methylmalonyl-CoA mutase. (i) **First DEAE-cellulose.** Crude extract was made into a slurry with DEAE-cellulose which had been previously equilibrated with 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM DTE as previously described (6). The resulting slurry was stirred for 40 min and then filtered through a Büchner funnel with Whatman no. 1 filter paper. DEAE-cellulose on the filter was washed with one volume of the Tris hydrochloride buffer containing 0.1 M KCl. The enzyme was subsequently eluted with one column volume of Tris hydrochloride buffer containing 0.15 M KCl. The eluted enzyme was concentrated by precipitation with ammonium sulfate (90% saturation), and the precipitate was dissolved in a minimum volume of 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM DTE.

(ii) **B₁₂-coenzyme affinity column.** The enzyme from the above step was stirred at 4°C for 45 min with a 1-ml settled volume of B₁₂-coenzyme Sepharose gel which had been previously equilibrated with 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM DTE. The mixture was poured into a column, and the unadsorbed material was washed out at a flow rate of 0.2 ml/min. Washing of the column and elution of the enzyme were done essentially as described previously (9). The column was washed with 200 volumes of 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM

DTE and 1 M NaCl and subsequently with 10 volumes of the same buffer containing 0.05 M NaCl and 0.01 M KCN. The column was left for 12 h in the same buffer, and then the column was washed with three column volumes of elution buffer consisting of 50 mM Tris hydrochloride (pH 8.0) containing 1 M NaCl and 3 mM B₁₂-coenzyme. The column was left in this buffer for 12 h, and then the enzyme was eluted with three column volumes of the elution buffer. The fractions containing the highest enzymatic activity were pooled, and the molarity of the buffer was reduced to 0.05 M by repeated dilution and concentration by ultrafiltration with a PM 10 membrane filter (Amicon Corp., Lexington, Mass.).

(iii) **Second DEAE-cellulose.** The concentrated enzyme solution described above was passed through a 1-ml bed volume of DEAE-cellulose which had been equilibrated with 0.05 M Tris hydrochloride buffer (pH 8.0) containing 1 mM DTE. The enzyme was eluted by washing the column with 5 ml of 50 mM Tris hydrochloride containing 0.15 M KCl. The fractions containing enzymatic activity were pooled and concentrated as described above.

Enzyme assay. The enzyme assay was done by measuring the incorporation of label from methylmalonyl-CoA into the permanganate-stable product as described previously (4). The reaction mixture contained 100 mM Tris hydrochloride buffer (pH 8.0), 0.5 mM [methyl-³H]methylmalonyl-CoA, 1 mM DTE, 60 µM B₁₂-coenzyme, and the enzyme in a total volume of 0.5 ml. After incubation of the reaction mixture containing all components except methylmalonyl-CoA for 10 min at 30°C, the reaction was initiated by the addition of methylmalonyl-CoA and allowed to proceed in the dark for 15 min. The reaction was terminated by the addition of 0.1 ml of 2 N perchloric acid. After being boiled for 5 min, the precipitated materials were removed by centrifugation at 10,000 × *g* for 5 min. A 0.3-ml sample of the supernatant was boiled for 10 min with 50 µl of 2 N perchloric acid, and 0.5 ml of 4% permanganate and the mixture was centrifuged. To 0.4 ml of the supernatant was added 0.25 ml of 99% propionic

FIG. 1. High-pressure liquid chromatographic analysis of erythromycin produced by *S. erythreus* growing in the presence of [1,4-¹⁴C,2,3-³H]succinate. The experimental details are given in the text. ●, Number of disintegrations per minute, representing ¹⁴C content per 1-ml fraction; ----, UV absorption due to coinjected authentic erythromycin.

acid, and the mixture was placed in a glass vial and completely dried under a low heat. The residue was dissolved in 0.1 ml of water, and the radioactivity was measured. The enzyme activity is expressed as micromoles of succinate formed per milligram of protein per minute.

Determination of radioactivity and protein. Radioactivity was determined by liquid scintillation spectrometry in a Tri-Carb U60-CD spectrometer (Packard Instrument Co., Inc., Rockville, Md.) equipped with a disintegrations-per-minute converter with 10 ml of ScintiVerse (Fisher Scientific Co., Pittsburgh, Pa.) as the scintillation fluid. In the dual-labeling studies, the $^3\text{H}/^{14}\text{C}$ ratios were determined by the appropriate program of the scintillation spectrometer. Protein was measured by the method of Lowry et al. (12), with bovine serum albumin as a standard.

Electrophoresis. Sodium dodecyl sulfate disk gel electrophoresis was performed in an analytical electrophoresis apparatus (Hoefer Scientific Instruments, Inc., San Francisco, Calif.) by the method of Laemmli (10). Electrophoresis was done in a gel tube (0.45 cm in diameter) system consisting of a 7.5% polyacrylamide resolving gel (7.5 cm, pH 8.9) and a 2.5% stacking gel (2.5 cm, pH 7.2), each containing 0.1% sodium dodecyl sulfate. Usually, 10 to 20 μg of the enzyme was heated in a boiling-water bath with 50 μl of Laemmli's sodium dodecyl sulfate buffer (10) and applied to the gel. Electrophoresis was performed at a constant current of 2 mA per tube over a period of 3 h. Protein bands were fixed and stained by Coomassie brilliant blue for 15 h. Destaining of the gel was performed with an Ames model 1801 quick gel destainer with 7.5% acetic acid and 5% methanol.

FIG. 2. Time course of appearance of methylmalonyl-CoA mutase (●—●) and erythromycin (○—○) production by *S. erythreus*. The experimental details are given in the text.

RESULTS

Incorporation of succinate into erythromycin. In an attempt to determine whether succinyl-CoA generated from carbohydrates, which are known to support excellent antibiotic production, provides methylmalonyl-CoA for erythromycin synthesis, we first tested whether doubly labeled succinate was incorporated in vivo into erythromycin. The substrate prepared by mixing $[1,4-^{14}\text{C}]$ succinate and $[2,3-^3\text{H}]$ succinate had a $^3\text{H}/^{14}\text{C}$ ratio of 0.9. After 12 h of incubation of the substrate with *S. erythreus*, the ethyl acetate extract of the extracellular fluid contained 0.74% of the ^3H label added to the culture. Thin-layer chromatographic analysis revealed that about 10% of the radioactivity contained in this extract was in the erythromycin fraction. Further analysis of this fraction by high-pressure liquid chromatography showed that the major part of the radioactivity was contained in a single component which cochromatographed with authentic erythromycin (Fig. 1). The $^3\text{H}/^{14}\text{C}$ isotopic ratio of this labeled erythromycin was 2.0, suggesting that the substrate was probably incorporated with the loss of one of the two labeled carbon atoms but with little loss in ^3H atoms.

Demonstration of methylmalonyl-CoA mutase activity in cell-free extracts. Since the results of the above-described experiments were consistent with the conclusion that succinate is converted to methylmalonyl-CoA, we examined the cell-free extract prepared from *S. erythreus* for its ability to convert succinyl-CoA to methylmalonyl-CoA. After incubation of the cell-free extract with $[1,4-^{14}\text{C}]$ succinyl-CoA, the protein was removed and the products were subjected to high-pressure liquid chromatography. After short periods of incubation (15 min), the principal labeled products were methylmalonyl-CoA (19%) and an organic acid fraction (57%) which was not retained in the column. Cochromatography of the label with authentic methylmalonyl-CoA confirmed the identity of the product. A much smaller quantity of label was detected in a fraction which cochromatographed with propionyl-CoA (2%). Since crude extracts of *S. erythreus* is known to contain a decarboxylase specific for the (*R*)-isomer of methylmalonyl-CoA, the formation of propionyl-CoA from succinyl-CoA via (*R*)-methylmalonyl-CoA should be expected. The above results demonstrated the presence of methylmalonyl-CoA mutase in the *S. erythreus* extract.

Correlation between mutase activity level and erythromycin production. If the methylmalonyl-CoA mutase activity detected in the cell-free extract of *S. erythreus* is involved in erythromycin production, the level of this activity might be expected to correlate with the rate of erythromycin production. Therefore, the time course of appearance of the mutase activity and that of erythromycin production were determined. In the sucrose-alanine medium, which allows production of excellent levels of antibiotics without growth, the most rapid production of erythromycin was observed between 24 and 48 h after the cells were transferred to this medium (Fig. 2). The mutase activity increased rapidly over this period of time. Initially, the mutase level was low, and during the 48-h period during which erythromycin production was rapid, the mutase level increased by more than 12-fold. As the antibiotic production leveled off, the enzyme level decreased dramatically (Fig. 2), revealing an excellent correlation between the activity level of the mutase and erythromycin production. Similar experiments were done with cultures growing in the vegetative medium. In this case, the antibiotic production started after 2 days of growth and reached the maximum by day 4. The mutase activity level showed excellent correlation with the rate of antibiotic

production; an 11-fold increase in total activity and a 4-fold increase in specific activity occurred by day 4, and subsequently the enzymatic activity decreased drastically (data not shown).

Purification of methylmalonyl-CoA mutase. Since methylmalonyl-CoA mutase appears to play a role in erythromycin production, we attempted to purify and characterize this enzyme. We used 48-h cultures because the mutase level was found to be maximal at that time. As soon as crude extracts were prepared, they were applied to DEAE-cellulose at pH 8.0, and the mutase was retained on this column. The activity could be recovered by elution with 0.15 M KCl with little loss in enzyme activity, resulting in an increase of about twofold in specific activity (Table 1). There was no significant loss of activity when this preparation was concentrated by ammonium sulfate precipitation. When the enzyme solution was applied to a B₁₂-coenzyme Sepharose affinity column, about half of the applied enzymatic activity passed through with the majority of the protein. The remaining half of the enzymatic activity was retained by the column. There was no detectable enzymatic activity present in the different washing steps. The enzyme was eluted from the column with B₁₂-coenzyme with low recovery (1.3 to 1.6% of the applied activity). The enzyme solution was applied to DEAE-cellulose at pH 8.0, which resulted in all the enzymatic activity being retained. Batch elution of the enzyme with 0.15 M NaCl resulted in 1.2% overall recovery of the enzymatic activity and 57-fold purification (Table 1). Polyacrylamide disk gel electrophoresis of the enzyme after treatment with sodium dodecyl sulfate and 2-mercaptoethanol showed a major band (Fig. 3). The subunit molecular weight of the enzyme, as determined by a linear plot of log molecular weight versus mobility of standard proteins, was 63,000. The enzyme was relatively unstable; it lost about 20 to 30% of the activity when stored for 2 days in the dark at -10°C in 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM DTE.

Effect of protein, incubation time, and pH on enzyme activity. The rate of formation of succinyl-CoA by the mutase increased with increasing protein concentration in a rectilinear manner at least up to 60 µg/ml, and the reaction rate was linear up to at least 30 min (Fig. 4). The enzyme showed a pH optimum of around 7.5. All subsequent experiments were done within linear range for both protein and time.

Effect of substrate and coenzyme concentrations. The purified enzyme was stimulated two- to threefold by the addition of exogenous B₁₂-coenzyme; increasing the concentration of the coenzyme increased the activity, giving a saturation pattern. The maximal activity was observed at about 12 µM B₁₂-coenzyme, and higher concentrations did not have any further effect on the rate. Increasing concentrations of methylmalonyl-CoA gave increasing rates, and a typical Michaelis-Menten type saturation pattern was obtained (Fig.

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme (10 µg) on a 7.5% polyacrylamide gel.

5). Double reciprocal plots were linear for both the substrate and B₁₂-coenzyme, and from these plots the *K_m*s were calculated to be 0.31 mM and 0.09 µM for the methylmalonyl-CoA and B₁₂-coenzyme, respectively. The *V* was calculated to be about 0.46 µmol/min per mg of protein.

DISCUSSION

S. erythreus produces erythromycin, presumably from one molecule of propionyl-CoA and six molecules of methylmalonyl-CoA. The enzymology of the biosynthesis of this antibiotic is obscure, and very little is known about the intermediary metabolism in this organism (1). Early studies have demonstrated incorporation of propionate, methylmalonate, and succinate into erythromycin (5, 7). The source of propionate and methylmalonate used for the synthesis of erythromycin, however, remains unknown. The fact that carbohydrates constitute the main carbon source, which allows a high level of production of macrocyclic antibiotics, suggests that succinyl-CoA might be a major source for methylmalonyl-CoA. The data presented in this paper show that [1,4-¹⁴C,2,3-³H]succinate was in fact incorporated into erythromycin, probably with loss of one of the two labeled carbon atoms, but with little loss in tritium, thus resulting in the doubling of the ³H/¹⁴C isotopic ratio during the incorporation of the doubly labeled substrate into erythromycin. This result is consistent with the hypothesis that succinyl-CoA is converted to methylmalonyl-CoA by a mutase before incorporation into erythromycin. If succinate was incorporated into erythromycin via other metabolic pathways involving extensive degradation, most of the tritium would have been lost, and erythromycin derived from this doubly labeled succinate would have a lower ³H/¹⁴C isotopic ratio than that of succinate.

In agreement with the above conclusion was the finding that a cell-free extract of this organism contained methylmalonyl-CoA mutase activity. Upon incubation of the cell-free extract with [1,4-¹⁴C]succinyl-CoA in the presence of B₁₂-

TABLE 1. Purification of methylmalonyl-CoA mutase

Purification step	Total protein (mg)	Total activity (µmol/min)	Sp act (µmol/min per mg of protein)	Recovery (%)
Crude extract	2,205	18.10	0.0082	100
DEAE-cellulose	937	14.99	0.019	82.8
B ₁₂ -coenzyme Sepharose column				
DEAE-cellulose	0.473	0.22	0.47	1.2

FIG. 4. Effect of time and protein on the rate of isomerization of methylmalonyl-CoA to succinyl-CoA by the purified enzyme. Unless otherwise shown, all reaction mixtures contained 3.5 μ g of the enzyme and were incubated for 15 min as described in the text.

coenzyme, labeled methylmalonyl-CoA was formed. The purification of methylmalonyl-CoA mutase was greatly facilitated by the use of B_{12} -coenzyme Sepharose affinity chromatography. The yield of the enzyme was low, owing at least in part to the fact that only apoenzyme, but not holoenzyme, could bind to this affinity column and because of the long purification procedure. Nevertheless, the present purification scheme yielded enough enzyme for the determination of some of its properties. The mutase activity was stimulated by about 2.5-fold by the presence of B_{12} -coenzyme, as has been observed with human (9) and sheep (13) liver methylmalonyl-CoA mutase and in contrast to the *Propionibacterium shermanii* enzyme, which showed an absolute requirement for B_{12} -coenzyme (8). The present enzyme showed a much higher K_m for methylmalonyl-CoA than that observed with *P. shermanii* but only slightly higher than that observed with mammalian liver enzyme. The pH optimum of the present enzyme was similar to that observed for the enzymes from *P. shermanii* and sheep liver. The subunit molecular weight of *S. erythreus* mutase was slightly larger than that of *P. shermanii* and slightly smaller than that of the mammalian enzymes (9, 13). Thus, properties of the present mutase are generally similar to those previously studied from other organisms.

The relationship between the time course of appearance of the mutase and that of erythromycin synthesis suggested that the enzyme might play a role in the antibiotic production. A 12-fold increase in mutase activity occurred in association with the rapid synthesis of erythromycin without any growth of the organisms, and when the antibiotic production leveled off, the activity of the mutase decreased

markedly. These observations are consistent with the notion that succinyl-CoA is a major source of methylmalonyl-CoA, a major building block for erythromycin production. The positive correlation between the mutase activity level and the rate of erythromycin production was obvious when the organism was allowed to grow in the vegetative medium and when the antibiotic production occurred without significant growth in the alanine-sucrose medium. Propionyl-CoA, used as the initial reactant in the synthesis of the aglycone, may be produced by the decarboxylation of methylmalonyl-CoA generated from succinyl-CoA by the decarboxylase which has been purified from this organism (unpublished observations). If so, the observation that the decarboxylase is specific for (*R*)-methylmalonyl-CoA, which is the enantiomer generated by the mutase, is consistent with the above conclusion. However, other sources for methylmalonyl-CoA, such as carboxylation of propionyl-CoA by the enzyme previously isolated from this organism (6), must also be considered. It is possible that methylmalonyl-CoA used for the antibiotic synthesis arises from multiple sources.

Enzymology of erythromycin production is poorly understood because the enzymes present in cell-free extracts of this organism appear to lose their activity extremely rapidly (1). Thus, the initial activity levels of enzymes in such extracts are low, and classical protein fractionation procedures cause rapid decline of this already low activity. Rapid absorption of the extract on DEAE-cellulose, followed by elution of the desired enzyme, appears to provide fairly active preparations in the case of a carboxylase (6), decarboxylase (unpublished observations), and the present mutase. Thorough washing of the cells to remove proteases

FIG. 5. Effect of methylmalonyl-CoA and B_{12} -coenzyme concentrations on the rate of the reaction and double-reciprocal plots. The reaction mixtures contained 3.5 μ g of the enzyme, and assay conditions were as described in the text.

which adhere to the cells and inclusion of protease inhibitors in the buffer also helped in getting fairly active enzymes from this organism. Use of such precautionary measures allowed us to obtain cell-free synthesis of deoxyerythronolide B (unpublished results). Although the product identity was confirmed with thin-layer and two different high-pressure liquid chromatographic steps, the rate of synthesis of the macrocyclic lactone was extremely low. It is hoped that the type of techniques developed to isolate active enzymes such as the present mutase from this organism will help in developing a cell-free system catalyzing the synthesis of the macrocyclic lactone at usable rates. Such a system is necessary before significant progress can be made in our understanding of the enzymatic mechanism of biosynthesis of the macrocyclic lactone antibiotics.

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