catalytic subunits of *Chromatium* and spinach carboxylases.

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Crystallization and Partial Characterization of Prenyltransferase from Avian Liver†

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ABSTRACT: Prenyltransferase (EC 2.5.1.1) has been obtained from chicken liver in a stable crystalline form. The enzyme has been shown to be homogeneous by polyacrylamide gel electrophoresis at pH 8.4, and by electrophoresis in sodium dodecyl sulfate containing gels. Electrofocusing of the crystalline enzyme results in a single sharp protein peak with a p*I* of 5.72. The protein is a dimer of molecular weight 86,000 whose subunits were not resolved by gel electrophoresis in sodium dodecyl sulfate. Michaelis constants of 0.5 μM for both isopentenyl pyrophosphate and geranyl pyrophosphate are 3–20-fold lower than those found for prenyltransferase from yeast or pig liver (Eberhardt, N., and Rilling, H. C. (1974), *J. Biol. Chem.* (in press); Dorsey, J. K., Dorsey, J. A., and Porter, J. W. (1966), *J. Biol. Chem. 241,* 5353; Holloway, P. W., and Popjak, G. (1967), *Biochem. J. 104,* 57). The enzyme primarily synthesizes farnesyl pyrophosphate from dimethylallyl or geranyl pyrophosphate although some geranylgeranyl pyrophosphate is formed under certain conditions. This is the first preparation of a stable crystalline enzyme of sterol and terpene biosynthesis.

Prenyltransferase (EC 2.5.1.1) has been isolated in a substantially purified form from pig liver by Dorsey *et al.* (1966) and Holloway and Popjak (1967). More recently, Eberhardt and Rilling (1974) have reported the preparation of homogeneous prenyltransferase from *Saccharomyces cerevisiae.* In all instances the copurification of both dimethylallyl transferase and geranyl transferase activities was demonstrated. Thus, the dimethylallyl transferase reaction (the condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate to form geranyl pyrophosphate) and the geranyl transferase reaction (the condensation of geranyl pyrophosphate with isopentenyl pyrophosphate to form farnesyl pyrophosphate) appear to be the combined functions of a single enzyme. Unfortunately, the homogeneous enzyme obtained from *S. cerevisiae* was insufficiently stable to permit an experimental approach to the deter-

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mination of the number and specificity of catalytic sites on this protein.

With the intent of gaining further insight into the problem of specificity, the purification of prenyltransferase from avian liver was undertaken. This previously untried source has yielded a stable crystalline enzyme suitable for study, and we now report the purification and initial characterization of this enzyme.

Materials and Methods

1-[^{3}H]Isopentenyl pyrophosphate was synthesized enzymically from 5-[^{3}H]mevalonic acid (New England Nuclear) using the method described by Tchen (1963). 1-[^{14}C]Isopentenyl pyrophosphate was purchased from Amersham/Searle. Labeled isopentenyl pyrophosphate was diluted to the desired specific activity with unlabeled isopentenyl pyrophosphate generously provided by Dr. C. Dale Poulter (Department of Chemistry, University of Utah). Farnesyl, geranyl, and dimethylallyl pyrophosphates were prepared by the method of Cornforth and Popjak (1969).

Two assay procedures were followed. During purification of the enzyme, an assay utilizing 1-butanol extraction of products offered the advantage of shortened assay times and also proved quite reliable. For this assay, incubation mixtures contained in a volume of 0.1 ml, 850 nmol of potassium phosphate buffer (pH 7.0); 85 nmol of $MgCl_2$; 10 nmol of geranyl pyrophosphate; and 5 nmol of isopentenyl pyrophosphate of specific activity 9.05 or 6.34 Ci/mol for 1-[^{3}H]- or 1-[^{14}C]isopentenyl pyrophosphate, respectively. After the addition of enzyme, reaction mixtures were incubated for 15 min at 30° and were stopped by the addition of 1 ml of saturated sodium chloride solution (required to reduce the extent of isopentenyl pyrophosphate partitioning into the organic phase) and 1 ml of 1-butanol. After thorough mixing and brief centrifugation, 0.5 ml of the 1-butanol phase which contained the reaction products was mixed with 1 ml of absolute ethanol and 10 ml of toluene containing 0.4% Omnifluor (New England Nuclear) and counted in a Packard TriCarb scintillation spectrometer.

For kinetic studies, the method of Holloway and Popjak (1967) was used which measures the acid-labile products of the reaction. Each assay mixture contained in a total volume of 1 ml, 9 μmol of potassium phosphate buffer (pH 7.0); 0.9 μmol of $MgCl_2$; and the appropriate concentrations of geranyl, farnesyl, and isopentenyl pyrophosphates. Incubations were for 1 min at 37°. Data for the double reciprocal plots were fitted using a linear regression analysis program from Wang Laboratories, Inc. Apparent Michaelis constants were determined from the secondary plots of the reciprocals of apparent V_{max} against the reciprocals of fixed substrate concentrations. One unit of activity represented the incorporation of 1 nmol of isopentenyl pyrophosphate into product per min.

Molecular Weight Estimation by Chromatography on Sephadex G-200. A 1.5 × 55 cm column of Sephadex G-200 equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 0.1 M KCl was used for molecular weight estimation. A mixture of 10 mg of horse liver alcohol dehydrogenase (MW 83,000), 10 mg of rabbit muscle lactic dehydrogenase (MW 142,000), 10 mg of horse heart cytochrome *c* (MW 13,400), and 0.2 mg of avian liver farnesyl pyrophosphate synthetase was applied and eluted at a flow rate of 7.6 ml/hr.

Polyacrylamide Gel Electrophoresis. Bovine serum albumin, catalase, lactic dehydrogenase, and myoglobin were used as molecular weight standards for electrophoresis in sodium dodecyl sulfate containing gels according to the method of Weber *et al.* (1972) on 10% acrylamide and 0.26% bisacrylamide gels. Alkaline gels were run using a Tris–borate–EDTA (pH 8.4) system (Peacock *et al.*, 1965) with 7.5% acrylamide and 0.18% bisacrylamide gels. All gels contained 10 mM 2-mercaptoethanol. The gels were stained using 0.05% Coomassie Brilliant Blue in methanol, water, and glacial acetic acid (5:5:1). Gels were destained overnight in the same solvent.

Electrofocusing was performed in a 110-ml LKB electrofocusing column using a 1% solution of pH 5–7 range Ampholine in a sucrose gradient as described by Vesterberg (1971). The focusing was continued for 2 days with an applied voltage of 400 V.

Enzyme Purification. All procedures were performed at 4° unless otherwise stated. The standard buffer was 10 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Protein determinations were by the biuret method (Koch and Putnam, 1971) using bovine serum albumin as standard.

Fresh chicken liver (1.7 kg) was homogenized in 4 l. of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM EDTA, for 2 min in a Waring Blender. The supernate obtained by centrifugation at 13,200*g* for 40 min was filtered through cheesecloth. Ammonium sulfate (877 g) was added to the 4.2 l. of solution (36% saturation) which was then centrifuged at 13,200*g* for 45 min. To the 3.45 l. of supernate, 439 g of ammonium sulfate (57% saturation) was added, and after 15 min, the mixture was centrifuged at 13,200*g* for 30 min. The pellet was suspended in standard buffer to a final volume of approximately 750 ml. This solution was dialyzed against several 10-l. changes of standard buffer until the conductivity of the protein solution approached that of the buffer (approximately 1.2 mmhos). After centrifugation at 27,000*g* for 30 min, the protein solution was applied to a 5 × 54 cm column of DEAE-cellulose (Bio-Rad) previously equilibrated with standard buffer. The column was washed with buffer at a rate of 260 ml/hr until the absorbance at 280 nm subsided to 0.5 and was then developed with a linear gradient of 10 mM potassium phosphate to 82 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 mM 2-mercaptoethanol (8 l. total volume). Peak prenyltransferase activity emerged after about 2 l. of the eluent had been collected. Fractions of specific activity above 100 were combined, concentrated with an Amicon Diaflo apparatus fitted with a PM-30 membrane, and then dialyzed against standard buffer without EDTA, until a conductivity of 1.2 mmhos was reached. The protein was loaded onto a 1.5 × 38 cm column of hydroxylapatite which was washed with 12 mM potassium phosphate buffer (pH 7.0) until the absorbance of the eluate at 280 nm subsided to 0.05. The column was developed with a linear gradient of 12 mM potassium phosphate buffer (pH 7.0) to 60 mM potassium phosphate buffer (pH 7.0), 10 mM in 2-mercaptoethanol (1 l. total volume) at a rate of 40–50 ml/hr. Combined active fractions were concentrated as above and dialyzed against standard buffer. Neutral saturated ammonium sulfate was added to the protein solution until a slight cloudiness appeared. The solution was then allowed to stand at room temperature for 4–5 hr, during which time the enzyme crystallized. After recrystallization, the enzyme crystals were suspended in saturated neutral ammonium sulfate

TABLE I: Purification of Avian Liver Prenyltransferase.

Fraction	Units[a] ($\times 10^{-4}$)	Protein (mg)	Specific Activity	Yield (%)	-Fold Purification
Crude supernate	41.6	2.28×10^5	1.8		
Ammonium sulfate	21.0	5.03×10^4	4.2	50	2.3
Dialysis and centrifugation	20.2	3.69×10^4	5.5	49	3.1
DEAE-cellulose chromatography	9.05	363	250	22	139
Hydroxylapatite chromatography	4.82	42.0	1148	12	638
2nd crystallization	2.15	17.6	1220	5.2	678

[a] These values are not corrected for isopentenyl pyrophosphate isomerase which is present in early stages of purification. Assays of isomerase activity are not reliable since they include a variable prenyltransferase contribution dependent upon the ratio of the two enzymes and time. About 30% of the initial activity may represent isomerase. This value drops to less than 20% after ammonium sulfate and is not significant after DEAE chromatography.

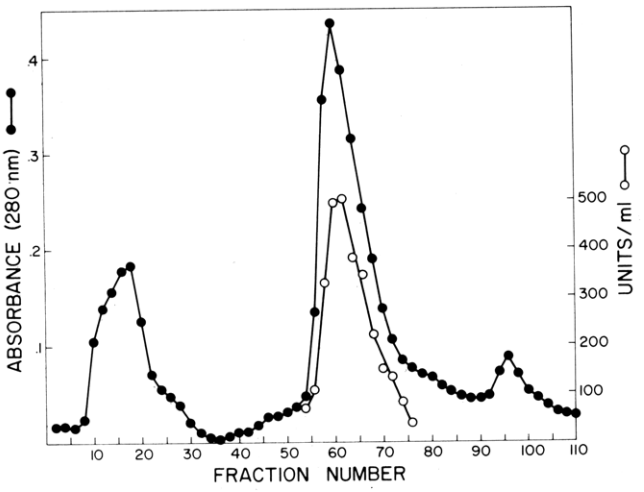

FIGURE 1: Hydroxylapatite column chromatography of protein obtained from DEAE-cellulose chromatography. The gradient was initiated at fraction 31; 5-ml fractions were collected.

and stored at 4°. As a crystalline suspension, the enzyme is stable for at least 2 months.

Results

A typical purification scheme is shown in Table I. The major purification step was chromatography on DEAE which gave approximately 45-fold enrichment; thus only one additional step, hydroxylapatite column chromatography, was required to obtain enzyme of 95% purity as judged by gel electrophoresis. A relatively flat specific activity curve was obtained through the active protein peak on hydroxylapatite chromatography (Figure 1), and only a slight increase in specific activity was obtained after repeated crystallization. Crystals obtained from the second crystallization are shown in Figure 2. The average long axis dimension is 40 μ, with the larger crystals approaching 80 μ in length. Electrophoresis of the crystalline protein in alkaline or sodium dodecyl sulfate gel systems shows only a single band of protein (Figure 3). Under nondenaturing conditions, enzyme activity migrated with the protein band. On isoelectric focusing, the crystalline protein displayed a single sharp peak in the range of focusing with no increase in specific activity. In fact, the specific activity decreased slightly, which we attribute to the absence of sulfhydryl protecting reagents during the run. The measured p*I* was 5.72.

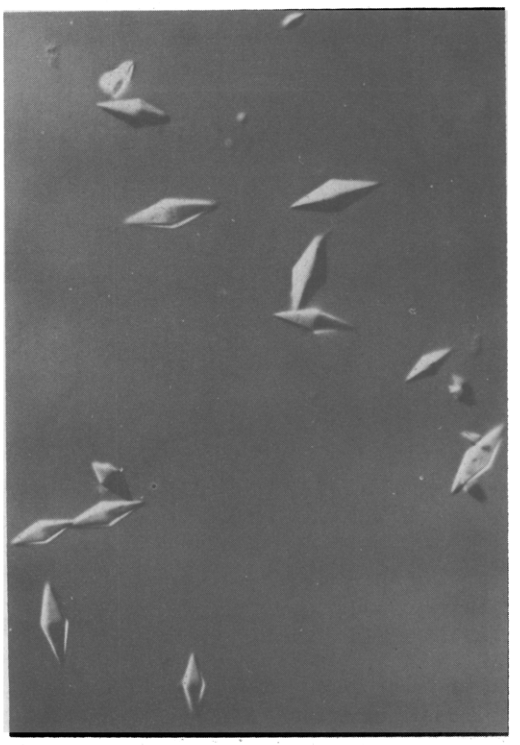

FIGURE 2: A photomicrograph of crystalline avian liver prenyltransferase. A Zeiss photomicroscope II was used with interference contrast optics.

Product analysis by chromatography on buffered silica gel "H" thin-layer plates (Sofer and Rilling, 1969) indicated that when the enzyme was incubated with dimethylallyl pyrophosphate and isopentenyl pyrophosphate the predominant product was farnesyl pyrophosphate as anticipated. Small amounts of geranyl pyrophosphate were consistently recovered from the reaction mixture. Similar incubations of farnesyl pyrophosphate and isopentenyl pyrophosphate with a 200-fold higher enzyme concentration (0.05–0.1 mg/ml) resulted in the synthesis of geranylgeranyl pyrophosphate, as has been previously observed with the homogeneous yeast enzyme (Eberhardt and Rilling, 1974).

Michaelis constants for isopentenyl pyrophosphate and geranyl pyrophosphate were determined as described under Materials and Methods over the concentration ranges shown in Figure 4. Each point is the average of five separate determinations. Essentially parallel lines were obtained

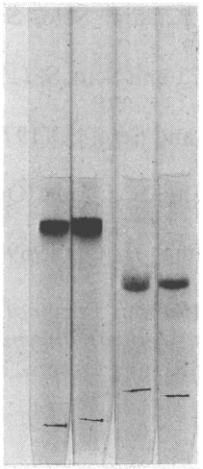

FIGURE 3: Polyacrylamide gels of crystalline prenyltransferase. From left to right: 17 and 34 μg of protein subjected to electrophoresis in 7.5% acrylamide gels at pH 8.4, and 10 and 20 μg of protein in 10% acrylamide gels containing sodium dodecyl sulfate.

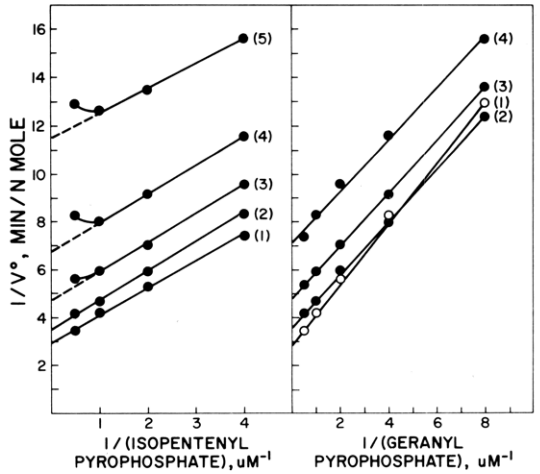

FIGURE 4: Lineweaver and Burk plots of farnesyl pyrophosphate synthesis from geranyl pyrophosphate and isopentenyl pyrophosphate. Fixed substrate concentrations were: 2 μM (1), 1 μM (2), 0.5 μM (3), 0.25 μM (4), and 0.125 μM (5) for geranyl pyrophosphate (left) and isopentenyl pyrophosphate (right). Each incubation mixture contained 132 ng of protein.

with Lineweaver and Burk plots. However, the points generated by the highest isopentenyl pyrophosphate concentration (2 μM) do show some apparent substrate inhibition which was more extreme at concentrations of isopentenyl pyrophosphate greater than those presented. The Michaelis constants of isopentenyl and geranyl pyrophosphates were both 0.5 μM. Similar studies utilizing farnesyl pyrophosphate and isopentenyl pyrophosphate as cosubstrates gave Michaelis constants of 8 and 18 μM, respectively. A V_{max}/E_0 = 3200 units/mg for the incorporation of isopentenyl pyrophosphate and geranyl pyrophosphate into farnesyl pyrophosphate, and a V_{max}/E_0 = 24 units/mg for the synthesis of geranylgeranyl pyrophosphate from farnesyl pyrophosphate were obtained.

When the molecular weight was determined by gel filtration, linear plots of log molecular weight against the distribution coefficients for the marker proteins were obtained. Prenyltransferase eluted with horse liver alcohol dehydrogenase whose MW is 83,000. Consequently, this value was taken as the best estimate for the molecular weight of the native enzyme.

Subunit molecular weight estimates by electrophoresis in sodium dodecyl sulfate containing gels were made from the log molecular weight *vs.* relative mobility plots from three runs. Only one band was discernible in prenyltransferase preparations (Figure 3), the mobility corresponding to that of a protein of molecular weight 42,900 ± 2140 (standard deviation). Thus, apparently the enzyme is composed of two identical or nearly identical subunits.

Discussion

This purification of chicken liver prenyltransferase represents the first preparation of a stable crystalline enzyme of terpene and sterol biosynthesis. Prenyltransferase has been purified to homogeneity from yeast also, but this enzyme was unstable and satisfactory crystals were not obtained. The molecular weights of the two enzymes are similar (84,000 for yeast and 86,000 for liver), and each is constituted of two subunits which are as yet indistinguishable. However, the enzymes have markedly different Michaelis constants for the substrates. The chicken liver enzyme has Michaelis constants for isopentenyl and geranyl pyrophosphates of 0.5 μM, which are 3–20-fold lower than those found for either the yeast or pig liver enzymes (Holloway and Popjak, 1967; Dorsey *et al.*, 1966; Eberhardt and Rilling, 1974).

Since prenyltransferase catalyzes the condensation of two different allylic pyrophosphates with isopentenyl pyrophosphate, the finding that the enzyme has two subunits emphasizes the questions of the number of catalytic sites and of their substrate specificity. Ogura, Seto, and their collaborators have presented data relevant to this problem. These workers prepared a series of analogs of dimethylallyl pyrophosphate with hydrocarbon chains of varying lengths extending trans from the double bond. Analogs whose size resembled either dimethylallyl pyrophosphate or geranyl pyrophosphate were active as substrates for prenyltransferase while the others tested were not (Nishino *et al.*, 1972). They found preferential protection of the geranyl transferase activity to heat denaturation in the presence of geranyl pyrophosphate (Ogura *et al.*, 1969). In other experiments, this group found that the monophosphate of dimethylallyl alcohol inhibited the utilization of dimethylallyl pyrophosphate but not geranyl pyrophosphate by this enzyme (Ogura *et al.*, 1969). Finally, an analog of isopentenyl pyrophosphate, 4-methylpent-4-enyl pyrophosphate, was utilized as a substrate with geranyl pyrophosphate but not dimethylallyl pyrophosphate (Ogura *et al.*, 1974). These experiments provide good evidence for independent catalytic sites for the two condensation reactions and in combination with our finding that one enzyme catalyzes both reactions raises the strong possibility that prenyltransferase contains two catalytically distinct sites. The presence of two subunits is consistent with a two-catalytic site hypothesis; however, we have been unable to resolve the subunits by means of electrophoresis in sodium dodecyl sulfate containing gels. Alternate approaches are in progress.

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Studies on the Binding of Acylaminoacyl-tRNA to Rat Liver 60S Ribosomal Subunits and Its Participation in the Peptidyltransferase Reaction†

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ABSTRACT: Peptidyltransferase with rat liver 60S subunits can be measured by the reaction between exogenous acylaminoacyl-tRNA and puromycin to form acylaminoacyl-puromycin in the presence of 33% methanol, 0.3 M KCl, and 4 mM $MgCl_2$. An assay system has been developed that allows examination of the binding of acetylphenylalanyl-tRNA to the ribosomal subunit "P" site, the transpeptidation of the 60S-bound substrate to puromycin, and the requirements for these individual steps. Binding of acetylphenylalanyl-tRNA to 60S subunits is stimulated several-fold by the addition of methanol, but the extent of binding in alcohol is the same in 60 as in 300 mM KCl containing solutions. Formation of acetylphenylalanyl-puromycin from 60S · acetylphenylalanyl-tRNA complex and puromycin stringently requires alcohol and the initial rate of the reaction is markedly greater at 300 mM KCl than at 60 mM KCl concentrations. Thus, alcohol and high concentrations of monovalent cation affect the reaction of an event subsequent to the binding of substrate to the "P" site. Preincubation of 60S subunits with poly(U), which stimulates the overall peptidyltransferase reaction, does not affect the amount of acetylphenylalanyl-tRNA that is bound to the particles; however, it markedly stimulates the initial rate of the transpeptidation reaction between 60S · acetylphenylalanyl-tRNA complex and puromycin. The codon specificity and the failure to affect binding with poly(U) suggest a role for the polynucleotide in the alignment or stabilization of the acylaminoacyl-tRNA on the "P" site rather than an effect on binding to either of the two particle sites or on the peptidyltransferase "active center." The effect of 40S subunits, which inhibit the overall peptidyltransferase reaction, on the binding of substrate could not be clearly interpreted since all three preparations, 60S subunits, 40S subunits, and combinations of 60S plus 40S particles, appear to bind acetylphenylalanyl-tRNA in the presence of methanol. However, the initial rate of peptide bond formation is several times greater with 60S · acetylphenylalanyl-tRNA complex than with 60S plus 40S particles containing bound acetylphenylalanyl-tRNA, and the addition of 40S subunits to preformed 60S · acetylphenylalanyl-tRNA complex during the transpeptidation phase of the reaction in methanol does not affect the rate of peptide bond formation. Thus, 40S subunits seem to inhibit peptidyltransferase by forming less reactive particles in aqueous solutions. Two inhibitors of peptidyltransferase, trichodermin and anisomycin, do not affect binding of substrate to the "P" site, but inhibit a subsequent step in the reaction with 60S-bound substrate.

The formation of peptide bonds in protein synthesis appears to be a property of the ribosomal particle and does not require protein factors or GTP (see review by Lucas-Lenard and Lipmann, 1971). This activity is associated with the large subunit of prokaryote or eukaryote ribosomes (Monro, 1967; Maden *et al.*, 1968; Monro *et al.*, 1969; Vazquez *et al.*, 1969; Falvey and Staehelin, 1970; Ballesta *et al.*, 1971; Nierhaus and Montejo, 1973) and can be assayed by reaction between exogenous donor molecules such as acylaminoacyl-tRNA or acylaminoacyl-oligonucleotide and acceptor molecules such as puromycin, aminoacyl-tRNA, or aminoacyl-oligonucleotide (Monro and Marcker,

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