Substrate Binding of Avian Liver Prenyltransferase†

Brent C. Reed‡ and Hans C. Rilling*

ABSTRACT: Prenyltransferase (farnesyl pyrophosphate synthetase) was purified from avian liver and characterized by Sephadex and sodium dodecyl sulfate gel chromatography, peptide mapping, and end-group analysis. The enzyme is 85 800 ± 4280 daltons and consists of two identical subunits as judged by sodium dodecyl sulfate gel electrophoresis, peptide mapping, and end-group analysis. Chemical analysis of the protein revealed no lipid or carbohydrate components. Avian prenyltransferase synthesizes farnesyl pyrophosphate from either dimethylallyl or geranyl pyrophosphate and isopentenyl pyrophosphate. A lower rate of geranylgeranyl pyrophosphate synthesis from farnesyl pyrophosphate and isopentenyl pyrophosphate was also demonstrated. Michaelis constants for farnesyl pyrophosphate synthesis are 0.5 μM for both isopentenyl pyrophosphate and geranyl pyrophosphate. The V_{max} for the reaction is 1990 nmol min^{-1} mg^{-1} (170 mol min^{-1} mol^{-1} enzyme). Substrate inhibition by isopentenyl pyrophosphate is evident at high isopentenyl pyrophosphate and low geranyl pyrophosphate concentrations. Michaelis constants for geranylgeranyl pyrophosphate synthesis are 9 μM for farnesyl pyrophosphate and 20 μM for isopentenyl pyrophosphate. The V_{max} is 16 nmol min^{-1} mg^{-1} (1.4 mol min^{-1} mol^{-1} enzyme). Two moles of each of the allylic substrates is bound per mol of enzyme. The apparent dissociation constants for dimethylallyl, geranyl, and farnesyl pyrophosphates are 1.8, 0.17, and 0.73 μM, respectively. Dimethylallyl and geranyl pyrophosphates bound competitively to prenyltransferase with one-for-one displacement. Four moles of isopentenyl pyrophosphate was bound per mole of enzyme. Citronellyl pyrophosphate, an analogue of geranyl pyrophosphate, was competitive with the binding of 2 of the 4 mol of isopentenyl pyrophosphate bound. The data are interpreted to indicate that each subunit of avian liver prenyltransferase has a single allylic binding site accommodating dimethylallyl, geranyl, and farnesyl pyrophosphates, and one binding site for isopentenyl pyrophosphate. In the absence of an allylic pyrophosphate or analogue, isopentenyl pyrophosphate also can bind to the allylic site.

Prenyltransferase (EC 2.5.1.1) catalyzes the synthesis of farnesyl pyrophosphate from isopentenyl pyrophosphate and either of two allylic substrates, dimethylallyl pyrophosphate or geranyl pyrophosphate. Substrate analogues have been utilized by several investigators in attempts to determine the specificity of the catalytic site(s) responsible for the two condensations. Popjak et al. (1969a) demonstrated that the primary binding determinant of the allylic substrate is the pyrophosphate group and that the stereochemistry and chain length of the hydrocarbon moiety only had secondary effects on the extent of substrate interaction with the catalytic site. Further experiments revealed that prenyltransferase can utilize allylic pyrophosphates with a wide variety of hydrocarbon chain lengths, providing these chains are not extensively branched. Thus, the region of the catalytic site which binds the allylic substrate probably has a polar pocket for the pyrophosphate moiety and a narrow hydrophobic groove for the hydrocarbon moiety (Popjak et al., 1969b; Ogura et al., 1970). After an extensive analysis of prenyltransferase activity with substrate analogues, Ogura suggested that the enzyme possesses two distinct catalytic sites, one for shorter substrates (dimethylallyl transferase site) and one for longer substrates (geranyl transferase site) (Nishino et al., 1972, 1973). The hypothesis for two separate sites was attractive since prenyltransferase from yeast or avian liver is comprised of two subunits (Eberhardt and Rilling, 1975; Reed and Rilling, 1975). However, definitive evidence for two distinct catalytic sites with different specificity is lacking and it is possible that the catalytic site accommodates either allylic substrate.

With the availability of a pure and stable prenyltransferase (Reed and Rilling, 1975), questions concerning the number and specificity of the catalytic site(s) in prenyltransferase could be approached directly. We now report the measurement of the binding of substrates to enzyme and a more thorough physical and chemical characterization of the enzyme.

Materials and Methods

Preparation of Substrates. [1-^{14}C]Isopentenyl pyrophosphate was prepared as described previously (Reed and Rilling, 1975). [1-^{3}H]Farnesol, -geraniol, and -dimethylallyl alcohol were synthesized by reduction of the corresponding aldehydes with NaB^3H_4. *trans,trans*-Farnesol was prepared by spinning-band distillation of an isomeric mixture obtained from Fluka, AG/Buchs, SG of Switzerland. Geraniol from Columbia Organic Chemicals Co. was distilled prior to use, and dimethylallyl alcohol was obtained through the generosity of Dr. W. W. Epstein (Department of Chemistry, University of Utah). Allylic pyrophosphates were prepared by phosphorylation of the respective alcohols by the method of Cornforth and Popjak (1969) and were purified by ion-exchange chromatography on Dowex AG 1-X8, formate form (Bio-Rad), using a linear ammonium formate gradient in 10% water in methanol (Sofer and Rilling, 1969). When possible, the unlabeled substrates were purified by crystallization. Dimethylallyl pyrophosphate was crystallized from methanol. Geranyl, citronellyl, and farnesyl pyrophosphates were crystallized from acetone–water mixtures. Unlabeled and labeled substrates were stored in water or 50% water–methanol, respectively. All compounds

† From the Department of Biochemistry, University of Utah College of Medicine, Salt Lake City, Utah 84132. *Received March 26, 1976.* This investigation was supported by Public Health Service Research Grant No. AM 13140 from the National Institute of Arthritis, Metabolism and Digestive Diseases. Taken in part from a dissertation submitted by B.C.R. to the Graduate School of the University of Utah, in partial fulfillment of the requirements for the Ph.D. degree.

‡ Present address: Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

were stored at −10 °C and maintained under basic conditions by the addition of aqueous ammonia.

Because of the lability of allylic pyrophosphates, frequent monitoring of their purity was necessary. Despite repeated purification by ion-exchange and preparative thin-layer chromatography, the allylic pyrophosphates were contaminated by several percent of the allylic mono- and triphosphates. We are at a loss to explain the appearance of these contaminants.

Determination of Concentration and Specific Activity of Substrates. The concentration of substrates was determined by analysis of total phosphate by a modification of the method of Richards and Boyer (1965). Hydrolysis of allylic pyrophosphates to inorganic phosphate was achieved by heating the sample with perchloric acid in a boiling water bath for 30 min. Error in the determination of total phosphate was less than 10%. Since isopentenyl and citronellyl pyrophosphates are acid stable, it was necessary to wet ash these samples in perchloric acid before phosphate determination.

Relatively small amounts of the labeled allylic pyrophosphates were available and, as a consequence, it was difficult to rule out the presence of inorganic pyrophosphate by chromatographic methods. Thus, independent methods were used to confirm the specific activities. Radioactive farnesyl pyrophosphate was prepared from each allylic substrate by incubation with avian liver prenyltransferase and [1-^{14}C]isopentenyl pyrophosphate. The labeled farnesol, liberated subsequently by treatment with alkaline phosphatase, was isolated by gas chromatography and the $^{3}H/^{14}C$ ratio determined. The specific activity of the allylic substrate was calculated from the established specific activity of [1-^{14}C]isopentenyl pyrophosphate. A similar method involved acetylation of [1-^{3}H]geraniol with [1-^{14}C]acetic anhydride of known specific activity and determination of the $^{14}C/^{3}H$ ratio of the resulting ester.

These two methods gave specific activities for the allylic compounds that differed by only 15% from those obtained by phosphate determinations. Since the difference was not in the direction anticipated if the substrates had been contaminated with inorganic pyrophosphate, the specific activities obtained by phosphate determination were used for subsequent calculations. The specific activities of the substrates were [1-^{3}H]dimethylallyl pyrophosphate (120 μCi/μmol), [1-^{3}H]-geranyl pyrophosphate (119 μCi/μmol), and [1-^{3}H]farnesyl pyrophosphate (405 μCi/μmol). The [1-^{14}C]isopentenyl pyrophosphate used in enzyme assays was 10 μCi/μmol, and that used in binding studies was 53 μCi/μmol.

Enzyme Assays. For kinetic studies, the method of Holloway and Popjak (1967) was used which measured the acid-labile products of the reaction. Each assay mixture contained in a total volume of 1 ml: 10 mM potassium phosphate buffer, pH 7.0; 1 mM $MgCl_2$; 10 mM 2-mercaptoethanol; and the appropriate concentrations of isopentenyl and geranyl or farnesyl pyrophosphate. Incubations were for 1 min at 37 °C, and less than 10% of the substrate was converted to product. For assays during enzyme purification, the mixture was modified to contain 5 μM [1-^{14}C]isopentenyl pyrophosphate and 25 μM geranyl pyrophosphate. Incubations at 37 °C were for 5 min. One unit of activity represents the incorporation of 1 nmol of isopentenyl pyrophosphate into product per min.

Radioisotope Determinations. Alcohols and hexane-soluble acid hydrolysis products were analyzed for radioactivity in 10 ml of toluene, containing 0.4% Omnifluor (New England Nuclear). The allylic pyrophosphates were dissolved in 1 ml of water and 10 ml of a 1:2 mixture of Triton X-100 (New England Nuclear) and toluene containing 0.85% Omnifluor. Double isotope determinations were obtained by standard techniques. In all cases counting efficiencies were determined by internal standardization.

Preparation of Enzyme. The enzyme was purified from avian liver by the method of Reed and Rilling (1975). Pure protein, assayed at 37 °C, had a specific activity between 1900 and 2100. The crystals were stored as a suspension in buffered saturated ammonium sulfate solution containing dithiothreitol at 4 °C. Under these conditions, the enzyme was stable for over a 4-month period.

Electrofocusing. Gel isoelectric focusing in 7.5% polyacrylamide gels containing 8 M urea and pH 3.5–10 range ampholine solution was run according to Wrigley (1971). Urea solutions were deionized by stirring over Amberlite MB-3 ion-exchange resin.

Polyacrylamide Gel Electrophoresis. Electrophoresis in sodium dodecyl sulfate-containing gels (10% acrylamide and 0.26% bisacrylamide) was according to the method of Weber et al. (1972). Alkaline gels were run in 8 M urea using a Tris[1]-borate–EDTA, pH 8.4, system with 5% acrylamide gels (Peacock et al., 1965).

Carboxymethylation. After reduction with 2-mercaptoethanol, protein was carboxymethylated in 8 M by the method of Crestfield et al. (1963).

Dry Weight Extinction Coefficient. Approximately 2 mg of crystalline protein was dissolved in 0.2 M ammonium formate adjusted to pH 7.0 with ammonium hydroxide and dialyzed against the same buffer overnight. The protein was then dialyzed against deionized water. The absorbance at 280 nm was determined and a portion was placed in a platinum boat, lyophilized, and maintained over P_2O_5 at 70 °C until a constant weight was obtained. After ignition in a gas–oxygen flame, the ash weight was determined.

Carbohydrate Determination. The carbohydrate content of the enzyme (0.6 mg of protein) was determined by the phenol–sulfuric acid method of Dubois et al. (1956) with glucose as standard. A 1% carbohydrate content by weight would have been detected.

Lipid Determination. Protein crystals (2 mg) were dissolved in 0.5 ml of water and then were extracted with 2.5 ml of a chloroform–methanol solution (2:1). After drying over anhydrous sodium sulfate and concentration of solvent under a stream of nitrogen, one-fifth of the solution was applied to a silica gel G plate. The plate was then either exposed to iodine vapors or sprayed with 50% concentrated sulfuric acid in methanol and charred at 115 °C. Fifteen micrograms of phosphatidylethanolamine was easily detected by these methods; therefore, 1.5% lipid content by weight would have been detected.

Amino Acid Analysis. Amino acid analyses were by a 5-h dual-column procedure (Beckman Instruction Manual AIM-2). Duplicate samples were hydrolyzed at 110 °C for 24, 48, 72, and 96 h. Carboxymethylcysteine content was determined in duplicate on reduced and carboxymethylated protein by hydrolysis of approximately 200 μg of protein in 1 ml of HCl at 110 °C for 24 and 48 h. Carboxymethylcysteine content was normalized to the values for the previous analyses by averaging the correction factors obtained from a comparison with the values of aspartic acid, proline, glycine, alanine, methionine,

[1] Abbreviations used are: Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

tyrosine, and phenylalanine for native or carboxymethylated protein.

Peptide Mapping. The modification of the method of Katz et al. (1959) as described by Kempe et al. (1974) was used. A known weight (between 1.5 and 3.0 mg) of carboxymethylated protein was suspended in 0.4 ml of 0.1 M ammonium bicarbonate buffer, pH 8.7. Trypsin (Tos-PheCH_2Cl-treated, 75% protein by weight, Worthington) was added at 0, 2, 4, and 6 h and the mixture incubated at 30 °C for a total of 10 h. The trypsin added was 1.5% of the protein by weight. After lyophilization, 3 N ammonium hydroxide was added to the digest and 3 μl (60 μg/μl) was spotted on 20 × 20 cm Eastman "chromagram" cellulose thin-layer sheets (No. 6064) and dried at room temperature. Ascending chromatography was with 1-butanol–glacial acetic acid–water (12:3:5) as solvent. The thin-layer sheet was again dried at room temperature and Whatman 3 MM filter paper wicks of appropriate lengths were sewn on the edges in preparation for transverse electrophoresis in a Gilson electrophoresis apparatus. Electrophoresis was at 1400 V, 11–18 mA, for 60 min at 10 °C, using a buffer of pyridine–glacial acetic acid–water (1:10:289). Peptides were detected by dipping the chromatogram in buffered ninhydrin solution and then heating for 15–20 min at 70 °C (Whitaker, 1967).

End-Group Determination. Samples (250- and 500-μg) of performic oxidized protein (Hirs, 1967) were subjected to dansylation according to the method of Gray (1972). Hydrolysis was in sealed ampules at 103 °C in 100 μl of constant-boiling HCl. Identification of the released N-terminal dansyl amino acid was accomplished by two-dimensional chromatography on polyamide thin-layer sheets (Chen-Chin layers, Pierce Chemical Company) using the solvent systems recommended by Gray (1972) and Hartley (1970).

Sulfhydryl Determinations Using Nbs_2. Total cysteine and disulfide bond content of the enzyme was determined as described by Habeeb (1972).

Binding Studies. Measurement of substrate binding by equilibrium dialysis was not possible because of the enzyme's ability to hydrolyze the allylic cosubstrates (Poulter and Rilling, 1976). To minimize hydrolysis during the binding measurements, a "forced dialysis" technique was used at 4 °C (Paulus, 1969; Cantley and Hammes, 1973). The method is rapid, and with the apparatus used, eight separate measurements could be made within 30 min of mixing substrate and enzyme. These precautions eliminated any major error resulting from substrate hydrolysis. All binding studies utilized 50 mM potassium Tes buffer, pH 7.0, containing 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 100 mM KCl. In each experiment, 100 μl of the appropriate concentration of ligand was equilibrated at 4 °C, 100 μl of enzyme was added, and the solution was transferred to one cell of the apparatus. Five microliters of solution was forced through the membrane by nitrogen at 25 psig and was collected from the bottom surface of the membrane (Spectrapor 2, Spectrum Medical Industries) with a 5-μl disposable capillary pipet. This sample was discarded to avoid errors by sample dilution from the previously damp membrane. Nitrogen pressure was again applied for 5 to 6 min. A 10-μl sample was then taken using Lang-Levy pipet (10 μl ± 1%) and the radioactivity analyzed to determine the free substrate concentration. The total substrate concentration was determined by sampling 10 μl from the top compartment prior to the "forced dialysis". The apparent concentration of the bound ligand was calculated by subtracting the free from the total concentration of ligand. The membrane contribution to apparent binding was a linear function approximately 3% of the free substrate concentration. The bound and free substrate concentrations observed were corrected for membrane retention errors using the empirically determined correction factors for each substrate.

For these studies, crystalline enzyme was pelleted, then dissolved in 100 μl of buffer, and desalted on a 0.5 × 25 cm Sephadex G-25 column within 30 min of the start of an experiment. The method resulted in a 7.5% to 10% concentration of enzyme during the measurement. The known enzyme concentration prior to "forced dialysis" was used for calculations.

Results

The molecular weight of avian liver prenyltransferase, determined by sodium dodecyl sulfate gel chromatography, is 85 800 (Reed and Rilling, 1975). The extinction coefficient at 280 nm as determined by dry weight, was 1.03 ± 0.05 ml mg^{-1} cm^{-1}, giving a molar extinction coefficient of 88 400 ± 6150 l. mol^{-1} cm^{-1}. Protein concentrations were calculated using this coefficient. Residual ash weight and lipid and carbohydrate content were insignificant.

Catalytic Properties of Prenyltransferase. Prenyltransferase synthesizes farnesyl pyrophosphate and small amounts of geranylgeranyl pyrophosphate (Reed and Rilling, 1975; Eberhardt and Rilling, 1975). For the synthesis of farnesyl pyrophosphate, the apparent K_m's for isopentenyl and geranyl pyrophosphate are both 0.5 μM. V_{max} for the reaction is 1990 units mg^{-1} (170 mol min^{-1} mol^{-1}). For the synthesis of geranylgeranyl pyrophosphate, the apparent K_m's for isopentenyl and farnesyl pyrophosphate are 20 and 9 μM, respectively. V_{max} for this reaction is 16 units mg^{-1} (1.4 mol min^{-1} mol^{-1}). The latter kinetic constants differ slightly from those reported earlier (Reed and Rilling, 1975) since the specific activity of [1-^{14}C]isopentenyl pyrophosphate used was in error.

Allylic Substrate Binding to Prenyltransferase. Preliminary substrate binding experiments revealed the enzyme hydrolyzed its allylic substrates autocatalytically. Although this rate, at its maximum, was only 0.1% of the normal catalytic rate, it necessitated the use of forced equilibrium dialysis at 4 °C for determination of substrate binding. Tes buffers were used instead of phosphate to eliminate possible stimulation of substrate hydrolysis by phosphate. Tes does not inhibit the enzyme.

The binding of [1-^{3}H]dimethylallyl pyrophosphate to avian liver prenyltransferase is presented as a Scatchard plot (Scatchard, 1949) in Figure 1A. The curve approximates that expected for ligand binding to two equivalent sites per enzyme dimer when the apparent dissociation constant is 1.8 μM. Binding of substrate was measured at other protein concentrations. The data generated diverging lines with the same intercept on the abscissa and lower intercepts on the ordinate. The results obtained for the binding of [1-^{3}H]geranyl pyrophosphate to prenyltransferase are presented in Figure 1B. Again, the Scatchard plot is linear and extrapolates to a saturation of 2 mol of geranyl pyrophosphate bound per mol of enzyme dimer. The dissociation constant obtained was 0.17 μM and is an order of magnitude smaller than that for dimethylallyl pyrophosphate. Here again, divergent lines were observed at other protein concentrations. The Scatchard plot for the binding of [1-^{3}H]farnesyl pyrophosphate to the enzyme (Figure 1C) is linear and extrapolates to a saturation of 2 mol of farnesyl pyrophosphate bound per mol of enzyme dimer. The apparent dissociation constant is 0.73 μM.

The apparent dependence of the dissociation constants of dimethylallyl and geranyl pyrophosphate on protein concen-

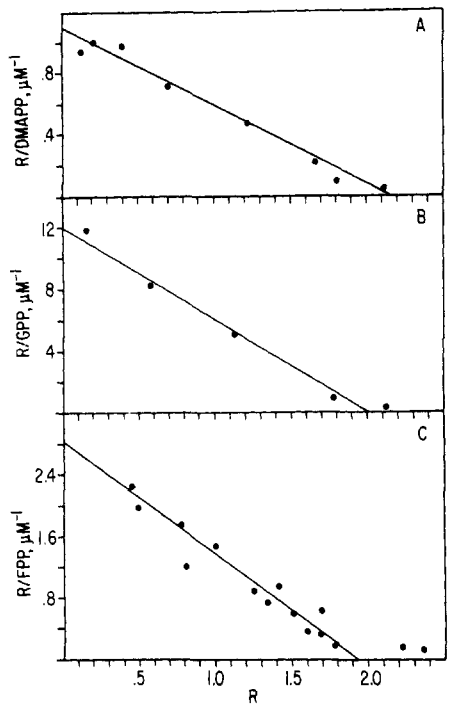

FIGURE 1: Binding of [1-^{3}H]allylic pyrophosphates, where R is the ligand bound per mole enzyme. DMAPP, GPP, and FPP are the free concentrations of dimethylallyl, geranyl, and farnesyl pyrophosphate, respectively. The enzyme concentration was 3.08 μM in A, 3.13 μM in B, and 5.80 μM in C.

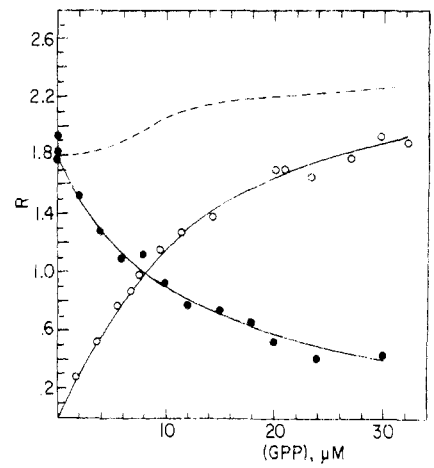

FIGURE 2: Competition between dimethylallyl and geranyl pyrophosphate for binding to avian liver prenyltransferase. R equals: moles of dimethylallyl pyrophosphate bound (●), moles of geranyl pyrophosphate bound (○), or moles of dimethylallyl pyrophosphate plus moles of geranyl pyrophosphate bound (- - -), per mole enzyme. Mixtures contained dimethylallyl pyrophosphate (20 μM), enzyme (5.53 μM), and varying concentrations of geranyl pyrophosphate as indicated.

tration could be indicative of protein-protein interactions. However, there are other ways to account for this observation. Although the enzyme catalyzed hydrolysis of the substrate would introduce a change in apparent binding constants dependent on enzyme concentration, the rate of hydrolysis required would be higher than that observed. Another explanation for the variation in binding constant would be the presence of impurities in the substrate. If the impurities did not bind to the enzyme, erroneously high estimates would be obtained for the free ligand concentration. The error propagated by impurities in the ligand would increase with increasing protein concentrations, because of the greater amount of substrate required to reach saturation. Despite repeated purification by chromatography, the allylic substrates contained approximately 5% impurities. In the experiments with dimethylallyl pyrophosphate, when the observed free ligand concentration was corrected for the presence of impurities, the data points fell on the same line, independently of enzyme concentration. In any event, the extrapolated saturation values are independent of the cause of the apparent variation in the dissociation constants.

Competitive Binding between Allylic Substrates. The avian enzyme was nearly saturated with [1-^{3}H]dimethylallyl pyrophosphate (20 μM, 90%), and the binding of this substrate was measured at various concentrations of geranyl pyrophosphate. In a similar experiment, the enzyme was 90% saturated with 20 μM dimethylallyl pyrophosphate, and the binding of [1-^{3}H]geranyl pyrophosphate was measured at various concentrations of [1-^{3}H]geranyl pyrophosphate. The number of moles of each ligand bound per mole of enzyme dimer is presented in Figure 2. The results show clearly that geranyl pyrophosphate and dimethylallyl pyrophosphate compete for the same site with a stoichiometry of 1 for 1. The total allylic pyrophosphate bound per dimer rose during the titration to a constant level of 2.1 mol per mol of enzyme dimer. Farnesyl pyrophosphate also displaces [1-^{3}H]dimethylallyl pyrophosphate from the enzyme. By adding farnesyl pyrophosphate (20 μM) to a mixture of enzyme and [1-^{3}H]dimethylallyl pyrophosphate (21 μM), the binding of [1-^{3}H]dimethylallyl pyrophosphate to enzyme was reduced from 85 to 40% of saturation. Increasing farnesyl pyrophosphate to 40 μM reduced the binding of dimethylallyl pyrophosphate to 25% of saturation. Competition between farnesyl and dimethylallyl pyrophosphates was not studied extensively because of the difficulty in controlling and estimating the concentration of unlabeled farnesyl pyrophosphate. We found that serial dilutions of [1-^{3}H]farnesyl pyrophosphate gave lower concentrations than anticipated. This effect was eliminated by dilution in the absence of Mg^{2+}, but the subsequent addition of Mg^{2+} resulted in a decrease of as much as 50% in the concentration of the [1-^{3}H]farnesyl pyrophosphate. Farnesyl pyrophosphate, since it is more hydrophobic than the other allylic substrates, may form micelles or might become oriented on glass or liquid surfaces. The binding data for [1-^{3}H]farnesyl pyrophosphate (Figure 1C) nevertheless is considered valid since concentrations of the ligand were determined by direct sampling of the final mixture.

The mutual competition and stoichiometry of binding observed with the three allylic pyrophosphates are consistent with one allylic binding site per monomer. The relative binding affinity of the allylic substrates decreases in the order: geranyl pyrophosphate > farnesyl pyrophosphate > dimethylallyl pyrophosphate.

Isopentenyl Pyrophosphate Binding. The presence of one allylic site per monomer implies the existence of one binding site per monomer for isopentenyl pyrophosphate. Since Holloway and Popjak (1967) demonstrated by kinetics that the allylic substrate was bound first, the binding of isopentenyl pyrophosphate by prenyltransferase was not anticipated in the absence of dimethylallyl or geranyl pyrophosphates. However, the enzyme bound 3.6 to 4.0 mol of isopentenyl pyrophosphate per mol of enzyme dimer at saturation (Figure 3). Two isopentenyl pyrophosphate molecules binding per monomer is difficult to reconcile with a single transferase site unless isopentenyl pyrophosphate is also interacting with the allylic site, which is consistent with the substrate inhibition shown by

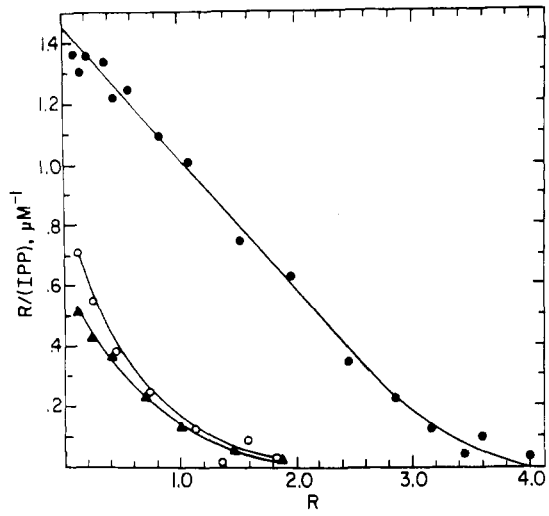

FIGURE 3: Binding of isopentenyl pyrophosphate to prenyltransferase in the presence and absence of citronellyl pyrophosphate. R is the number of moles of isopentenyl pyrophosphate bound per mole of enzyme, and (IPP) is the free concentration of isopentenyl pyrophosphate. Concentrations of citronellyl pyrophosphate were: 0 μM (●), 15 μM (○), and 50 μM (▲). The average enzyme concentration was 5.74 ± 0.09 μM.

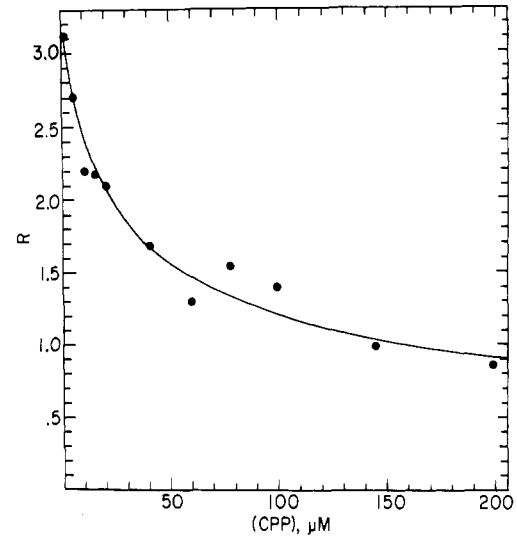

FIGURE 4: Competition between isopentenyl pyrophosphate and citronellyl pyrophosphate for binding to avian liver prenyltransferase. R is moles of isopentenyl pyrophosphate bound per mole of enzyme. Mixtures contained isopentenyl pyrophosphate (55 μM), enzyme (5.55 μM), and varying concentrations of citronellyl pyrophosphate as indicated.

isopentenyl pyrophosphate (Reed and Rilling, 1975).

If isopentenyl pyrophosphate were interacting with the allylic site, analogues of allylic substrates would bind competitively with isopentenyl pyrophosphate. Citronellyl pyrophosphate (2,3-dihydrogeranyl pyrophosphate) is such an analogue and is inactive as a substrate. Its inhibition constant (0.29 μM) with respect to geranyl pyrophosphate shows it binds efficiently to the allylic site (Popjak et al., 1969a). Prenyltransferase, 80% saturated with 55 μM [1-^{14}C]isopentenyl pyrophosphate, was incubated with citronellyl pyrophosphate at the concentrations shown in Figure 4. Binding of isopentenyl pyrophosphate was reduced to 2 mol per mol of enzyme dimer with 20 μM citronellyl pyrophosphate and, with increasing concentrations of citronellyl pyrophosphate, an additional mole of isopentenyl pyrophosphate was displaced. Scatchard plots of the data obtained for the binding of isopentenyl pyrophosphate at increasing concentrations of citronellyl pyrophosphate qualitatively indicate interaction of citronellyl pyrophosphate with half of the sites to which isopentenyl pyrophosphate is bound. The curvature of the plots of these data indicates that the interactions are more complex than a simple one-for-one displacement at the allylic site (Figure 3).

Subunit Similarities. The results of the binding experiments strongly suggest that the enzyme has two identical catalytic sites per mole of protein. This, of course, implies that the two subunits are identical. The following observations provide further evidence for this similarity. Attempts to demonstrate the existence of more than one subunit species by gel isoelectric focusing in urea, or by electrophoresis in alkaline gels containing urea, failed to show more than one component. Sodium dodecyl sulfate gel electrophoresis of carboxymethylated protein confirmed the presence of one major protein band, as was observed with unmodified protein in the same system (Reed and Rilling, 1975). End-group analysis revealed only one α-amino acid derivative, *N*-dansylvaline.

The amino acid analysis of the protein is presented in Table I. Residues of amino acid per 85 800 g of protein were normalized to 100% recovery. A separate analysis of reduced and carboxymethylated protein gave 12.9 carboxymethylcysteine residues per 85 800 g protein, which is in close agreement with the 12.6 mol of cysteine per mol of enzyme measured by Nbs_2 titration of the denatured enzyme. A comparison of sulfhydryl content before and after reduction of the denatured protein with 2-mercaptoethanol indicated no disulfide bonds were present.

TABLE I: Amino Acid Analysis of Avian Liver Prenyltransferase.

Amino Acid	Residue Recovered μmol	g of Residue[a] per 100 g Recovered	Residue[b] per 85 000 g of Protein
Lys	0.0354 ± 0.0011	8.24	55.3
His	0.0077 ± 0.0015	1.91	12.0
Arg	0.0285 ± 0.0005	8.08	44.5
Asp	0.0385 ± 0.0014	8.05	60.1
Thr[c]	0.0154	2.83	24.1
Ser[c]	0.0218	3.45	34.1
Glu	0.0750 ± 0.0016	17.6	117
Pro	0.0182 ± 0.0012	3.22	28.5
Gly	0.0335 ± 0.0001	3.47	52.3
Ala	0.0402 ± 0.0010	5.20	62.8
Half-Cystine[c]	0.0083 ± 0.0002	1.55	12.9
Val	0.0356 ± 0.0009	6.41	55.6
Met	0.0088 ± 0.0004	2.09	13.7
Ile	0.0180 ± 0.0004	3.71	28.2
Leu	0.0530 ± 0.0015	10.9	82.8
Tyr	0.0267 ± 0.0008	7.92	41.7
Phe	0.0202 ± 0.0005	5.40	31.5
Try	ND[d]		

[a] Eighty percent recovery from 0.284 mg of protein per hydrolysis. [b] Normalized to 100% recovery of amino acids. [c] Extrapolated to zero hydrolysis time. [d] Not determined.

Peptide mapping provided an alternate approach to detecting differences in subunits. Amino acid analysis gave an estimated total arginine plus lysine content of 100 ± 10 (2σ) residues per mole of enzyme. Tryptic digests of carboxymethylated protein would be expected to contain between 46 and 56 peptides if the subunits were identical and complete peptide resolution was achieved. Completely dissimilar subunits would yield about twice this number. Peptide maps obtained from two separate digests of carboxymethylated protein

consistently revealed 48 peptides, while an additional 10 were variable or lightly stained. Thus, there was a maximum of 58 detectable peptides, indicating no differences between the two subunits.

Discussion

The preparation of pure prenyltransferase from yeast and chicken liver permitted the demonstration that the two C_1 to C_4' condensations required for farnesyl pyrophosphate synthesis from dimethylallyl and geranyl pyrophosphates are catalyzed by a single enzyme (Eberhardt and Rilling, 1975; Reed and Rilling, 1975). However, the specificity and number of catalytic sites remained to be established. Ogura, Seto, and their collaborators approached this problem by several different methods. They examined a series of analogues of dimethylallyl pyrophosphate with hydrocarbon chains of various lengths extending trans from the double bond as substrates for the enzyme. Analogues whose size approximated either dimethylallyl pyrophosphate or geranyl pyrophosphate were maximally active as substrates (Nishino et al., 1973). In other experiments, they found preferential protection of geranyltransferase activity against heat denaturation by geranyl pyrophosphate (Ogura et al., 1969a). Also, this group demonstrated that the monophosphate of dimethylallyl alcohol inhibited the utilization of dimethylallyl pyrophosphate, but not geranyl pyrophosphate (Ogura et al., 1969b). Finally, an analogue of isopentenyl pyrophosphate, 4-methylpent-4-enyl pyrophosphate, was utilized as a substrate with geranyl pyrophosphate but not dimethylallyl pyrophosphate (Ogura et al., 1974). Their experiments point strongly to the existence of separate dimethylallyl and geranyltransferase sites. However, their experiments are not conclusive since some of their results might reflect differences in affinity displayed by a single allylic binding site toward the substrates and inhibitors employed. For example, the preferential inhibition of dimethylallyl transfer activity by dimethylallyl monophosphate was tested at a single substrate concentration. Marked differences in affinity of dimethylallyl pyrophosphate or geranyl pyrophosphate for the enzyme would result in preferential inhibition of dimethylallyl transfer by dimethylallyl monophosphate, even if there were only one binding site. The observation that 4-methylpent-4-enyl pyrophosphate condensed with geranyl pyrophosphate and not with dimethylallyl pyrophosphate may also be given an alternate interpretation. Although designed as an analogue of isopentenyl pyrophosphate, 4-methylpent-4-enyl pyrophosphate might also have competed at the allylic site. This would be consistent with the broad specificity of this site and the observed inhibition of the avian enzyme by isopentenyl pyrophosphate (Reed and Rilling, 1975). By finding preferential reactivity of the analogue with the more tightly bound geranyl pyrophosphate, they concluded a separate dimethylallyl transferase site existed, when in fact an enzyme with a single allylic binding site could have generated identical data. The strongest data presented for separate dimethylallyl and geranyl transferase sites were those from the preferential heat denaturation of the dimethylallyl transferase activity of the pumpkin enzyme in the presence of geranyl pyrophosphate. However, the pumpkin enzyme used by Ogura for this experiment was not pure, and therefore the presence of other prenyltransferases with differing specificities is possible.

The preparation of prenyltransferase from avian liver provided a stable enzyme to utilize for exploring the problem of the number and specificity of catalytic sites, and we have analyzed substrate binding as an approach to this problem. When saturated, prenyltransferase from avian liver bound 2 mol of any of the three allylic pyrophosphates per mol of enzyme (1 molecule bound per monomer) (Figure 1A–C). These sites were shown to be identical by the finding that dimethylallyl and geranyl pyrophosphates competed for the same binding site (Figure 2). The total number of sites occupied by both substrates maximized at 1.1 sites per monomer over a range of substrate concentrations which guaranteed 80% or greater saturation for either substrate in the absence of the other. Likewise, it was possible to demonstrate competition between farnesyl pyrophosphate and dimethylallyl pyrophosphate. This mutual competition for binding to the enzyme exhibited by the three allylic pyrophosphates establishes that the two subunits each possess one allylic binding site and that these sites are identical.

The apparent dissociation constants for dimethylallyl, geranyl, and farnesyl pyrophosphates are 1.8, 0.17, and 0.73 μM, respectively. The dissociation constant for geranyl pyrophosphate is an order of magnitude lower than for dimethylallyl pyrophosphate and accounts for the difficulty in detecting geranyl pyrophosphate after prolonged incubation of the enzyme with dimethylallyl pyrophosphate and isopentenyl pyrophosphate. However, the relatively low dissociation constant for the product, farnesyl pyrophosphate, is surprising, especially considering its poor reactivity as a substrate. Obviously, the end product of the enzyme is not determined by binding affinity, but rather by the ability of the substrate to align properly at the catalytic site.

One allylic binding site per monomer, in the simplest situation, would require one binding site for the cosubstrate isopentenyl pyrophosphate. The order of binding determined kinetically (Holloway and Popjak, 1967) predicts insignificant isopentenyl pyrophosphate binding in the absence of allylic substrate. However, binding experiments with isopentenyl pyrophosphate showed two molecules of isopentenyl pyrophosphate were bound per monomer (Figure 3). The similarity in structure between isopentenyl pyrophosphate and dimethylallyl pyrophosphate, as well as the observed substrate inhibition by isopentenyl pyrophosphate, strongly suggested that isopentenyl pyrophosphate was binding to the allylic site. This proposal is consistent with both the anticipated number of binding sites per monomer and the kinetic requirement for occupation of the allylic site prior to binding of isopentenyl pyrophosphate. Citronellyl pyrophosphate, a nonallylic analogue of geranyl pyrophosphate, was shown kinetically to interact only with those enzyme intermediates predicted to interact with an allylic pyrophosphate (Popjak et al., 1969a). This property of citronellyl pyrophosphate was used to demonstrate that once the allylic site is occupied, the enzyme binds a maximum of one molecule of isopentenyl pyrophosphate per monomer. At 15 and 50 μM concentrations of citronellyl pyrophosphate, the data obtained for isopentenyl pyrophosphate binding are very similar (Figure 3). This is a reasonable indication that the allylic site, where isopentenyl and citronellyl pyrophosphates compete for binding, is saturated with citronellyl pyrophosphate. In agreement with the proposal, isopentenyl pyrophosphate binding saturates at one molecule bound per monomer at these higher concentrations of citronellyl pyrophosphate.

In summary, avian liver prenyltransferase is most simply described as an enzyme of approximately 86 000 daltons with two identical subunits. Each subunit has one catalytic site.

Acknowledgment

The authors are grateful to Dr. K. Alexander for the preparation of radioisotopic substrates.

References

Cantley, L. C., and Hammes, G. G. (1973), *Biochemistry 12,* 4900.
Cornforth, R. H., and Popjak, G. (1969), *Methods Enzymol. 15,* 359.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238,* 622.
Dubois, M., Gelles, K. A., Hamilton, J. K., Rebers, D. H., and Smith, F. (1956), *Anal. Chem. 28,* 350.
Eberhardt, N. L., and Rilling, H. C. (1975), *J. Biol. Chem. 250,* 863.
Gray, W. R. (1972), *Methods Enzymol. 25,* 121.
Habeeb, A. F. S. A. (1972), *Methods Enzymol. 25,* 457.
Hartley, B. S. (1970), *Biochem. J. 119,* 805.
Hirs, C. W. (1967), *Methods Enzymol. 11,* 197.
Holloway, P. W., and Popjak, G. (1967), *Biochem. J. 104,* 57.
Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234,* 2897.
Kempe, T. D., Gee, D. M., Hathaway, G. M., and Noltman, E. A. (1974), *J. Biol. Chem. 249,* 4625.
Nishino, T., Ogura, K., and Seto, S. (1972), *J. Am. Chem. Soc. 94,* 6849.
Nishino, T., Ogura, K., and Seto, S. (1973), *Biochim. Biophys. Acta 302,* 33.
Ogura, K., Koyama, T., and Seto, S. (1969a), *Biochem. Biophys. Res. Commun. 35,* 875.
Ogura, K., Koyama, T., Shibuya, T., Nishino, T., and Seto, S. (1969b), *J. Biochem. (Tokyo) 66,* 117.
Ogura, K., Nishino, T., Koyama, T., and Seto, S. (1970), *J. Am. Chem. Soc. 92,* 6036.
Ogura, K., Saito, A., and Seto, S. (1974), *J. Am. Chem. Soc. 96,* 4037.
Paulus, H. (1969), *Anal. Biochem. 32,* 91.
Peacock, A. C., Bunting, S. L., and Queen, K. G. (1965), *Science 147,* 1451.
Popjak, G., Holloway, P. W., Bond, R. P. M., and Roberts, M. (1969a), *Biochem. J. 111,* 333.
Popjak, G., Rabinowitz, J. L., and Baron, J. M. (1969b), *Biochem. J. 113,* 861.
Poulter, C. D., and Rilling, H. C. (1976), *Biochemistry 15,* 1079.
Reed, B. C., and Rilling, H. C. (1975), *Biochemistry 14,* 50.
Richards, O. C., and Boyer, P. D. (1965), *J. Mol. Biol. 11,* 327.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Sofer, S. S., and Rilling, H. C. (1969), *J. Lipid Res. 10,* 183.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26,* 3.
Whitaker, J. R. (1967), in Paper Chromatography and Electrophoresis, Vol. 1, New York, N.Y., Academic Press, p 95.
Wrigley, C. W. (1971), *Methods Enzymol. 22,* 559.

Stereochemistry of the Reaction of Sheep Liver Threonine Dehydratase. A Nuclear Magnetic Resonance and Optical Rotatory Dispersion Study of Its Reaction Pathway and Products†

Gordon Kapke‡ and Leodis Davis*

ABSTRACT: Products, substrates, and inhibitors of the threonine dehydratase from sheep liver (EC 4.2.1.16) have been investigated by proton nuclear magnetic resonance and optical rotation. The α-ketobutyrates produced from L-threonine and L-allothreonine in ^2H_2O have been shown to incorporate a single deuterium into the β position. The dehydratase forms *R*-α-ketobutyrate-β-*d* from L-threonine and L-allothreonine. The α protons of the substrates, threonine and allothreonine, do not exchange in the presence of the dehydratase. In the presence of dehydratase, the competitive inhibitors L-cysteine and L-alanine undergo α-proton exchange. Highly purified dehydratase has been used to determine kinetic parameters for the substrates L-threonine, L-allothreonine, L-serine, and L-chloroalanine. L-Chloroalanine, in addition to being a substrate, inhibits the dehydratase in a manner kinetically identical with that of L-serine.

Sheep liver threonine dehydratase converts L-threonine to α-ketobutyrate and requires an α-ketobutyryl group as a cofactor rather than pyridoxal phosphate (Kapke and Davis, 1975). With this report and the one by Cohn and Phillips (1974) of a similar microbial serine–threonine dehydratase, further investigations of the mechanism by which keto acids function in dehydration is warranted. Presumably, keto acid cofactors function in catalysis by forming a Schiff base with the amino acid substrate and promoting α-proton labilization. A study of the chirality of the products was made to obtain information about the stereochemistry of the elimination reaction. The labilization of the α proton of substrates and inhibitors has been studied by proton nuclear magnetic resonance. The results presented here add support to the suggested

† From the Department of Chemistry, University of Iowa, Iowa City, Iowa 52242. *Received February 4, 1976.* This work was supported in part by Grant AM 16950 from the National Institutes of Health.

‡ The work in this paper forms part of the dissertation in partial fulfillment for the Degree of Doctor of Philosophy.