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HUMAN LIVER PRENYLTRANSFERASE AND ITS CHARACTERIZATION *

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Prenyltransferase (dimethylallydiphosphate : isopentenyldiphosphate dimethylallytransferase, EC 2.5.1.1) has been purified to homogeneity from human liver obtained at autopsy. The enzyme is a dimer with a native molecular weight of 74 000 ± 1 400. The amino acid composition is reported. The enzyme has a broad pH optimum between 7.3 and 8.8 and an absolute requirement for either Mn^{2+} or Mg^{2+} for activity; half-maximal activity was observed at 3.7 μM Mn^{2+} or 89.0 μM Mg^{2+}. Michaelis constants for geranyl pyrophosphate and isopentenyl pyrophosphate were 0.44 and 0.94 μM, respectively; the V value for synthesis of farnesyl pyrophosphate from these substrates was 1.1 μmol · min^{-1} · mg^{-1}. Isopentenyl pyrophosphate inhibited the reaction rates at concentrations above 2 μM when the concentrations of geranyl pyrophosphate were less than 2 μM. The highest concentration of geranyl pyrophosphate tested, 16 μM, showed no inhibition of reaction rates even when the concentration of isopentenyl pyrophosphate was as low as 0.2 μM. Only one form of human liver prenyltransferase could be observed under conditions which resolved the porcine enzyme into two distinct forms; the human enzyme is akin, physico-chemically, to the B-form of the pig liver enzyme. After dialysis against Tris-HCl buffer, pH 7.8, the enzyme became completely dependent upon dithiols or thiols for its activity. Kinetic experiments with a partially activated enzyme sample showed that the activation by the dithiol greatly enhanced the affinity of the enzyme for geranyl pyrophosphate, but not that for isopentenyl pyrophosphate. The human prenyltransferase is inactivated by phenylglyoxal according to pseudo-first-order kinetics, but is protected against the inactivation by 3,3-dimethylallyl and geranyl pyrophosphate. It is also inactivated by high concentrations (>2 mM) of iodoacetic acid, but is protected against the inactivation by dithiothreitol. Antibodies raised to the B-form of the pig liver enzyme cross-reacted with the human prenyltransferase and were 47% as effective in precipitating the human enzyme as the porcine enzyme. In double immunodiffusion experiments the antiserum was monospecific against the B-form of the porcine enzyme; it also gave a single precipitin line with the A-form, but not identical with that given by the B-form. It gave a precipitin line also with the human enzyme, but not identical with that given by either the A- or B-form of the porcine enzyme.

Introduction

Prenyltransferase (dimethylallydiphosphate : isopentenyldiphosphate dimethylallytransferase, EC 2.5.1.1) catalyzes the sequential, irreversible 1′-4 condensation of 3,3-dimethylallyl pyrophosphate and geranyl pyrophosphate with isopentenyl pyrophosphate to farnesyl pyrophosphate. The enzyme has been purified to homogeneity from yeast [1], pig liver [2,3] and was obtained in crystalline form from chicken liver [4]. Significant differences exist among these liver prenyltransferases. For example, the porcine enzyme exists in two separable forms

* This paper is dedicated to Professor David Shemin on his 70th birthday.

Abbreviations: SDS, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

[5,2], which are interconvertible [2] and represent two different oxidation-reduction states of the same protein [6,7], probably resulting from intramolecular disulfide bond formation and reduction [3,7]. The chicken liver enzyme apparently exists only in one form. Prenyltransferase has been proposed as an ideal target enzyme to achieve a controlled reduction of cholesterol biosynthesis [8], and although some inhibitors of prenyltransferase effective in vitro have been described [9,10], in view of the differences between the transferases from various sources we decided that a characterization of the human enzyme was an important prerequisite for further studies of inhibitors aiming at a reduction of plasma cholesterol levels in man.

We report here the first purification and characterization of an enzyme of sterol biosynthesis from a human source, that of liver prenyltransferase, its molecular weight, amino acid composition, conditions for its maximal activity, kinetic constants, effects of thiol activation and immunologic cross-reactivity with the pig liver enzyme. A brief description of some of the results has been given earlier [11,12].

Materials and Methods

Substrates. 3-Methyl[1-^{14}C]but-3-en-1-yl pyrophosphate ([1-^{14}C]isopentenyl pyrophosphate), specific activity 0.5 Ci/mol, geranyl pyrophosphate and the carrier prenols were the same preparations as used in this laboratory previously [10]. A specimen of [1-^{14}C]isopentenyl pyrophosphate with a specific activity of 56 Ci/mol was obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

Assay of prenyltransferase. The enzyme was assayed essentially by the method of Holloway and Popják [13] as detailed recently [3]. Routine assays to locate the enzyme during the purification contained (in 0.5-ml incubations) 16 μM [1-^{14}C]isopentenyl pyrophosphate, specific activity 0.5 Ci/mol, and 16 μM geranyl pyrophosphate. For kinetic studies with the pure enzyme the 0.5-ml incubations in 10 mM Tris-HCl buffer, pH 7.8/2 mM $MgCl_2$/2 mM dithiothreitol contained 7.8 ng enzyme and were carried out at 37°C for 10 min with varied substrate concentrations as indicated in the figures. In these assays the [1-^{14}C]isopentenyl pyrophosphate with the specific activity of 56 Ci/mol was used. The triplicate, or quadruplicate assays were terminated and worked up as described [3].

Purification of human liver prenyltransferase. Human liver, obtained 5 h post mortem, was frozen with solid CO_2, and kept frozen until needed. The frozen liver was broken into small pieces and homogenized for 30 s in a Waring Blendor in 100 g batches, each with 200 ml of 10 mM 2-mercaptoethanol in water, initially at 22°C. All subsequent operations were carried out at 4°C. The pH of a 3.7 l homogenate was adjusted from 7.1 to 5.2 with 3.5 N acetic acid. After being stirred for 1 h, the homogenate was centrifuged at 3 200 rev./min for 45 min. The supernatant was decanted through glass wool and the pellet resuspended in 1.3 l of 10 mM 2-mercaptoethanol, pH 5.2, and the suspension recentrifuged. The second supernatant was passed through glass wool and added to the first supernatant. Powdered $(NH_4)_2SO_4$ was added to the pooled supernatant to 47.5% saturation. After being stirred for 1 h, the precipitate was sedimented at 16 000 $\times g$ for 45 min and the supernatant discarded. The precipitate was resuspended in 10 mM sodium acetate buffer, pH 6.0/10 mM 2-mercaptoethanol and dialyzed overnight against the same buffer.

Batch treatment with carboxymethyl cellulose. The pH of the dialyzed preparation (32 mg protein/ml and 0.87 mΩ^{-1} conductivity) was adjusted to 5.0 by the addition, with stirring, of 3.5 N acetic acid. Whatman CM-52 cellulose (375 mg dry resin/30 mg protein), equilibrated for 15 min with 10 mM sodium acetate buffer, pH 5.0/5 mM 2-mercaptoethanol, was added to the protein solution with stirring. After 15 min the suspension was centrifuged at 6 000 rev./min for 10 min and the supernatant decanted and kept. The resin was washed twice by resuspension in 10 mM sodium acetate buffer, pH 5.0/5 mM 2-mercaptoethanol; after each centrifugation the supernatants were pooled and their pH adjusted to 6.0 with 1 N NaOH.

DEAE-cellulose column chromatography at pH 6.0. DEAE-cellulose (Whatman DE-52), equilibrated with 10 mM sodium acetate buffer, pH 6.0/10 mM 2-mercaptoethanol (167 mg wet weight/10 mg protein), was added to the enzyme sample from the previous step (3.3 mg/ml, and conductivity 0.75 mΩ^{-1}). The suspension was stirred for 15 min and

then the resin allowed to settle. An assay of the supernatant showed that 95% of the enzyme units were bound to the resin. The supernatant was discarded and the resin was washed three times with the sodium acetate buffer by decantation and then the resin was added to the top of a 1.6 × 75 cm column of DEAE-cellulose equilibrated with 10 mM sodium acetate buffer, pH 6.0/10 mM 2-mercaptoethanol, to give a final bed height of 87 cm. A linear gradient of 1 800 ml total volume from 0–140 mM $(NH_4)_2SO_4$ in the same buffer was applied after the column had been washed with 20 ml equilibrating buffer. Fractions of 14-ml were collected at a flow-rate of 1.5 ml/min and assayed for enzymic activity, protein content (A_{280}/A_{260}) and conductivity. Prenyltransferase was obtained in a single symmetrical peak centered at 5.3 $m\Omega^{-1}$ conductivity. The enzyme fractions were pooled, concentrated by pressure filtration though a PM-10 membrane (Amicon Corporation, Lexington, MA) at 50 lbs/inch^2 to about 5 ml and then this solution was dialyzed against 10 mM potassium phosphate buffer, pH 7.2/10 mM 2-mercaptoethanol.

Butyl-agarose column chromatography. A 1 × 60 cm column of butyl-agarose (Sigma Chemical Co., St. Louis, MO) was equilibrated with 10 mM potassium phosphate buffer, pH 7.2/10 mM 2-mercaptoethanol; the protein sample was applied at the top. After the column was washed with 80 ml of the equilibrating buffer, an 800-ml linear gradient of 10–60 mM potassium phosphate buffer, pH 7.2/10 mM 2-mercaptoethanol was applied at a flow-rate of 1 ml/min. Fractions of 8-ml were collected and assayed for enzymic activity, protein content and conductivity. Enzyme fractions were pooled and concentrated to 6 ml by pressure filtration.

Calcium phosphate column chromatography. A 1.5 × 85 cm column of calcium phosphate gel, freshly prepared by the method of Mathews et al. [14], was equilibrated with 10 mM Tes/KOH buffer, pH 7.0/10 mM 2-mercaptoethanol. The enzyme sample from the previous step was applied to the column which was then washed with 20 ml equilibration buffer before the application of a 1 600 ml linear gradient of 0–90 mM potassium phosphate buffer, pH 7.0, in the Tes buffer. Fractions of 13-ml were collected at a flow-rate of 1.5 ml/min and were assayed as before. The enzyme was stored as a precipitate in $(NH_4)_2SO_4$ solution at 60% saturation.

Polyacrylamide gel electrophoresis. The preparation of protein for SDS-polyacrylamide gel electrophoresis at pH 7.2 on 10% gels and the electrophoresis at a constant current of 8 mA/tube for 4 h were carried out as described by Weber et al. [15]. The molecular weight markers used were either the covalently cross-linked polymers of a monomer M_r = 14 300–57 200 (British Drug Houses, Ltd., No. 44 223; Poole, Dorset, U.K.). or the 'SDS-6' protein mixture of Sigma Chemical Company containing lysozyme (M_r = 14 300), β-lactoglobulin (M_r = 18 400), trypsinogen (M_r = 24 000), pepsin (M_r = 34 700), hen ovalbumin (M_r = 45 000) and bovine serum albumin (M_r = 67 000). Proteins were stained with Coomassie brillant blue R-250 (0.025% in 45.5% methanol/9.2% acetic acid) for 1 h; the gels were destained in 7.5% acetic acid/5% methanol in water containing AG-50W-X8 ion-exchange resin (Biorad, Richmond, CA). For the examination of the native enzyme, 10% polyacrylamide gels polymerized with $(NH_4)_2S_2O_8$ were made as described by Maurer (gel system 3) [16]. Gels were pre-electrophoresed overnight at 1 mA/tube before application of samples. At the end of the experimental electrophoresis (4 mA/tube for 3 h) the gels were stained with Coomassie G-250 (0.02% in 12.5% trichloroacetic acid). Duplicate unstained gels were cut into slices, 2.3-mm thick, which were then assayed in pairs for prenyltransferase by inclusion of the slices in the regular assay medium.

Sephacryl S-200 gel exclusion chromatography. The molecular weight of native prenyltransferase was determined according to the method of Andrews [17] on a 2.5 × 77 cm column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer, pH 7.0/50 mM KCl. The column was calibrated with yeast glucose-6-phosphate dehydrogenase (M_r = 102 000), bovine serum albumin (M_r = 67 000) and hen ovalbumin (M_r = 45 000). Samples were applied to the top of the column in 1.5 ml eluting buffer; 5-ml fractions were collected at a flow-rate of 21 ml/h. Fraction tubes were weighed before and after chromatography to determine precisely the elution volume of each protein. Fractions were assayed for enzymic activity (prenyltransferase and glucose-6-phosphate dehydrogenase), or for protein content by a modified method of Lowry et al. [18]. The void volume was

determined with blue Dextran and the total elution volume was determined for each run with tritiated water (Amersham-Searle, Arlington Heights, IL).

Tests for possible two forms of human prenyltransferase. The elution position of prenyltransferase from a 0.7 × 13 cm DE-52 column, equilibrated with 10 mM Tris-HCl buffer, pH 7.6, with or without 5 mM 2-mercaptoethanol, was determined as previously described [7]. A thiol-reduced sample of human prenyltransferase (155 μg) was dialyzed against 10 mM Tris-HCl buffer, pH 7.6/5 mM dithiothreitol overnight before application to the column equilibrated with this latter buffer. A 'thiol oxidized' sample of human prenyltransferase was prepared by the addition of neutral oxidized glutathione to 232 μg of the protein in 10 mM Tris-HCl buffer, pH 7.6, to a final concentration of 1.7 mM. The solution was stirred for 3 days at 4°C. The solution was applied to a 0.7 × 14 cm column of DE-52 equilibrated and eluted with 10 mM Tris-HCl buffer, pH 7.6, without thiol. A linear gradient of 0–150 mM $(NH_4)_2SO_4$ in the buffers was used for elution.

Thiol requirements for activity. 5 μl of a suspension of pure human prenyltransferase precipitated with $(NH_4)_2SO_4$ (1.55 mg/ml; 7.55 μg) were dissolved in 500 μl water and dialyzed for 2.5 h against 250 ml 100 mM Tris-HCl buffer, pH 7.8 (no thiol and no Mg^{2+}), and dialyzed for another hour against 250 ml 10 mM Tris-HCl buffer. Samples (5 μl) of the dialyzed enzyme were assayed for activity with 0.5 μM substrates; the additions of thiols were as indicated in the legends to figures. Assays were for 4 min and blank incubations contained no enzyme.

Metal ion requirements for activity. A sample of the pure human liver prenyltransferase ($(NH_4)_2SO_4$ precipitate; 1.55 mg/ml) was diluted 200-fold in 10 mM Tris-HCl buffer, pH 7.8, and used without dialysis. Assays were carried out in the presence of 2 mM dithiothreitol with 9.3 ng of the transferase/ml and additions of metal ions as shown in the legends to figures.

Effect of ionic strength on enzyme activity. Standard assays with 0.5 μM substrates for 10 min were performed with 2 mM dithiothreitol, 5 mM Mg^{2+}, 9.3 ng/ml prenyltransferase and various molarities of Tris-HCl buffer, pH 7.8, or KCl in 10 mM Tris-HCl buffer, pH 7.8.

Antibodies to prenyltransferase. Two New Zealand white rabbits were injected subcutaneously between the scapulae every 10 days with 1.2 ml of a sterile emulsion of a 1 : 1 mixture of Freund's complete adjuvant and the B-form of porcine transferase [3] (0.7 mg/ml) over a period of 50 days. Freund's incomplete adjuvant replaced the complete adjuvant after the first two injections. Serum samples were prepared from blood taken from an ear vein before each injection. The immunization was followed by the appearance of precipitin lines in double-immunodiffusion plates (Miles Laboratories, Elkhart, IN) developed at 4°C and by immunotitrations.

Immunotitration was used to quantify the antibody titer: 175-μl capacity Airfuge tubes (Beckman Instruments Inc., Palo Alto, CA) contained 115 μl 10 mM Tris-HCl buffer, pH 7.8, with 5 mM 2-mercaptoethanol, 126 ng prenyltransferase in 10 μl of 1 mM dithiothreitol, 0–10 μl antiserum and 10–0 μl control serum (obtained before immunization) to make up the volume of serum to a constant 10 μl. The components were mixed by a pasteur pipette and then left to stand on ice for 30 min before centrifuging at 132 000 × *g* for 20 min. The top two-thirds of the contents of each tube were transferred to separate tubes, the contents mixed, and 25 μl samples were assayed for prenyltransferase in 15-min incubations. The activity of the samples containing 10 μl of control serum and no antiserum was almost identical with that obtained without serum, indicating that the control (preimmune) serum caused no inhibition of the transferase.

Amino acid analysis. For determination of the amino acid composition of the human prenyltransferase, three experiments were carried out. In each, three samples of 175–200 μg enzyme were carboxymethylated and then hydrolyzed with 3.5 N mercaptoethanesulfonic acid for 24 h as we have described recently [3]. An average value for the decomposition of each amino acid after a 24-h hydrolysis was determined from the hydrolysis of porcine prenyltransferase under identical conditions [3]; this measure allowed the determination of the amino acid content at zero-time of hydrolysis. Cysteine was determined as carboxymethylcysteine. The average recovery of amino acids was 77% of theoretical relative to protein determinations before carboxymethylation and with bovine serum albumin as standard.

Inactivation by phenylglyoxal and iodoacetic acid. Experiments with phenylglyoxal, an arginine-modifying reagent, were carried out as described for the pig liver enzyme [3] except that the experiments, in 10 mM borate buffer at pH 8.2, were carried out with 2 mM phenylglyoxal. Experiments with iodoacetic acid (1–50 mM) were carried out in Tris-HCl buffer, pH 7.8, with and without 2 mM dithiothreitol.

Results

Purification of human liver prenyltransferase. The method of purification of prenyltransferase is based on those previously used in our laboratory [3] and on those of others [2], but with the inclusion of a hydrophobic chromatography step. The unusually high content of hydrophobic amino acid residues in porcine prenyltransferase [3] and the fact that the enzyme substrates contain hydrophobic moieties suggested that the human enzyme might be a good candidate for purification by hydrophobic chromatography. The binding of prenyltransferase from a crude liver extract to a set of C_2–C_8 alkyl-agaroses was tested. Butyl-agarose was found to retard the prenyltransferase without binding it too strongly. Thus a 4–6-fold purification could be rapidly achieved by the use of a butyl-agarose column from which the enzyme emerged just after the pass-through fraction, at the very beginning of the gradient and before the bulk of the protein. Hexyl- and octyl-agaroses bound the prenyltransferase very strongly and gave a poor recovery of the enzyme after application of a gradient of increasing ionic strength. Poor recovery of prenyltransferase of some prokaryotic preparations from longer chain alkyl-agaroses has been recently documented [19].

Data from one typical purification of the human enzyme are summarized in Table I. After the last step the enzyme appeared to be homogeneous as judged by SDS-polyacrylamide electrophoresis at pH 7.2 on a 10% gel, and also by electrophoresis of the native enzyme at pH 8.1. A single sharply-stained band was obtained after the SDS-polyacrylamide gel electrophoresis, whether the gel was lightly or heavily loaded (4–20 μg). The staining of the protein band after electrophoresis under the native conditions was a little diffuse, but the transferase activity coincided precisely with this protein band in duplicate unstained gels (Fig. 1).

Molecular weight and amino acid composition of human prenyltransferase. The molecular weight of the monomer of the enzyme was determined by SDS-polyacrylamide gel electrophoresis at pH 7.2 against two sets of standards. When the polymers of a cross-linked monomer, M_r 14 300–57 200, were the

TABLE I

PURIFICATION OF HUMAN LIVER PRENYLTRANSFERASE

Details of the purification steps are described under Materials and Methods. Specific activities are expressed as nmol farnesyl pyrophosphate synthesized from geranyl pyrophosphate and isopentenyl pyrophosphate (nmol · min^{-1} · mg^{-1} protein). The specific activity of the whole liver homogenate, before centrifugation, was 0.18 nmol · min^{-1} · mg^{-1}. All measurements of prenyltransferase activity were corrected for contamination by isopentenyl pyrophosphate isomerase by subtracting values obtained in assays with [1-^{14}C]isopentenyl pyrophosphate without geranyl pyrophosphate. This correction became negligible (<2%) after the first column chromatography.

Purification step	Volume (ml)	Total enzyme units	Total protein (mg)	Specific activity	Degree of purification	Yield (%)
1. Supernatant of homogenate [a]	3 740.0	26 910	52 950	0.5	2.8	100
2. $(NH_4)_2SO_4$ precipitate	238.0	19 515	7 567	2.6	14.3	73
3. CM-52 supernatant	429.0	16 961	1 650	10.3	57.0	63
4. DE-52 column eluate	40.0	12 748	232	54.9	305.0	47
5. Butyl-agarose column eluate	11.2	9 690	44	220.0	1 215.0	36
6. Calcium phosphate column eluate	3.6	7 373	8	922.0	5 122.0	27

[a] Prepared from 1.32 kg liver.

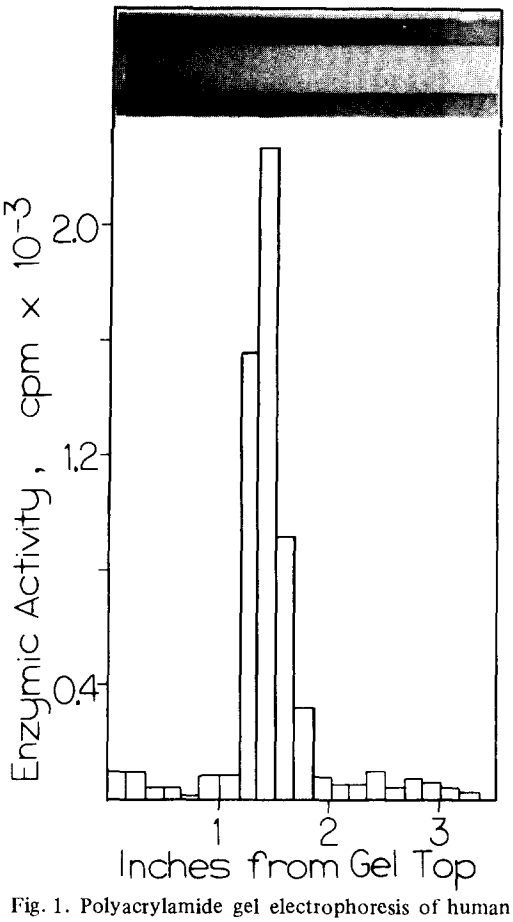

Fig. 1. Polyacrylamide gel electrophoresis of human prenyltransferase and localization of enzymic activity on the gel. The upper gel shows the single band of protein after SDS-polyacrylamide gel electrophoresis. The lower gel is from the electrophoresis of the native enzyme (about 10 μg). A duplicate unstained gel from the electrophoresis of the native enzyme was sliced and assayed for enzyme activity. The graph below the gels shows the coincidence of enzyme activity with the protein. The similar electrophoretic migration of the denatured and native enzyme is fortuitous.

standards, the human prenyltransferase gave an M_r of 39 500 ± 200 (four determinations) as compared to M_r = 40 800 ± 300 for the pig liver transferase. Measured against a set of natural protein standards (Sigma; SDS-6), the human prenyltransferase gave an M_r of 37 300 ± 1 200 (four determinations while the porcine enzyme gave an M_r of 38 500 ± 1 200. The latter value is the same as obtained previously [3]. Upon admixture of small amounts of human and porcine prenyltransferase (4–6 μg each per gel) followed by electrophoresis in the presence of SDS, the stained protein band was always broader than that obtained with either enzyme sample alone and this broadening could not be accounted for by a too high protein concentration. However, in only two out of five such experiments was there any suggestion of a separation between the enzyme from the two sources. From the two sets of determinations we calculate that the mean molecular weight of the subunits of the human prenyltransferase is 38 400 ± 1 220.

The molecular weight of the native form of the prenyltransferase was determined by gel exclusion chromatography on a Sephacryl S-200 column with glucose-6-phosphate dehydrogenase, bovine serum albumin and hen ovalbumin as standards. Each protein was run separately to eliminate the possibility of protein-protein interactions. The human prenyltransferase gave an M_r of 74 000 ± 1 400 consistent with a dimeric structure of the protein.

Throughout the purification only one form of human prenyltransferase was observed. This contrasts with the pig liver enzyme which exists in two forms, A and B [2,3,5,6]. These two forms are interconvertible their interconversion being governed by the state of thiol oxidation-reduction of the protein [7,3]. Using both crude, partially purified and freshly purified homogeneous preparations of the human enzyme under conditions which favored the thiol-reduction A-form and subsequently the thiol-oxidized B-form of the pig liver enzyme, we observed only one form of the human enzyme akin to the B-form of the porcine transferase by its elution position from a DEAE-cellulose column (Fig. 2).

The amino acid composition of the human liver prenyltransferase, shown in Table II, is remarkably similar to that of the pig liver enzyme [3]: it has a high content of hydrophobic residues and is poor in half-cystine.

Metal ion requirement of human prenyltransferase. Human liver prenyltransferase has an absolute requirement for either Mn^{2+} or Mg^{2+} for activity, the former ions having a much greater affinity for the enzyme than the latter. Mn^{2+} gave half-maximal activation at 3.7 μM, but Mg^{2+} only at 89.0 μM (Fig. 3); however, at saturating concentrations (Mn^{2+} at 20 μM and Mg^{+} at 2 mM) both ions gave equal maximum activity.

pH Optimum, effects of ionic strength and thiols on activity of human prenyltransferase. Among the

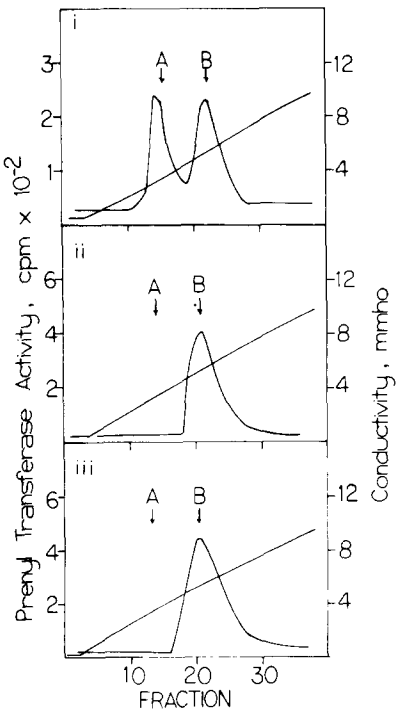

Fig. 2. Chromatography of porcine and human prenyltransferase on DEAE-cellulose columns. Test for possible existence of two forms of the human enzyme. In experiments (i) and (ii) 0.7 X 13 cm 'analytical' columns [7] were equilibrated with 10 mM Tris-HCl buffer, pH 7.6/5 mM 2-mercaptoethanol; in (iii) the 2-mercaptoethanol was omitted. The columns were eluted with a 300-ml linear gradient of 0–150 mM $(NH_4)_2SO_4$ in the equilibration buffers. (i) Mixture of the A- and B-forms of porcine prenyltransferase showing their characteristic elution positions [7], marked by arrows, at 3.5 $m\Omega^{-1}$ (A-form) and at 5.3 $m\Omega^{-1}$ (B-form). (ii) Human prenyltransferase pretreated with 2 mM dithiothreitol; (iii) the human enzyme after exposure to oxidized glutathione. In both (ii) and (iii) the peak of enzyme activity was eluted at 5.4 $m\Omega^{-1}$ conductivity.

various buffers tested, maximal activity was observed with Tris-HCl buffers with a broad pH optimum between 7.3 and 8.8. Phosphate buffers were distinctly inhibitory, as was noted for the porcine enzyme [13]. High ionic strength buffers were also severely inhibitory. For example, in 100 mM Tris-HCl buffer, pH 7.8, or 10 mM Tris-HCl buffer/100 mM KCl, the activity was only 76% and at 500 mM concentrations of the buffer, or of KCl in the dilute buffer, the activity was only 8–12% of the maximum observed in 10 mM Tris-HCl buffer. Assays aiming at maximum enzyme activity were carried out in 10 mM Tris-HCl buffer, pH 7.8, rather than the 100 mM buffer of the same pH commonly used with the pig liver transferase.

TABLE II

AMINO ACID COMPOSITION OF HUMAN PRENYLTRANSFERASE

The mean values were calculated per subunit M_r 38 400. For comparison the amino acid composition of porcine liver prenyltransferase, calculated for M_r 38 500, is also shown.

Amino acid	Residues per subunit	
	Human liver enzyme	Pig liver enzyme [a]
Cys (CM)	5.8	6.0
Asx	32.8	30.9
Thr	10.0	11.7
Ser	10.3	14.3
Glx	45.2	45.9
Pro	12.0	15.2
Gly	23.7	22.8
Ala	27.2	26.5
Val	20.9	21.2
Met	6.4	7.3
Ile	16.5	19.0
Leu	37.4	36.7
Tyr	17.4	17.3
Phe	14.2	16.0
Trp	2.0	4.6
Lys	21.8	21.1
His	4.4	5.5
Arg	17.5	17.5

[a] From Barnard and Popják [3].

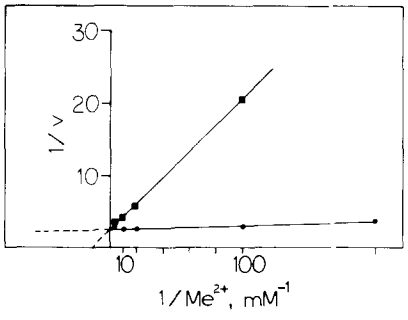

Fig. 3. Divalent metal ion (Me^{2+}) requirement of human prenyltransferase. Double-reciprocal plots of transferase activity ($1/v$, $\mu mol^{-1} \cdot min \cdot mg$) against the reciprocal of metal ion concentrations (mM^{-1}): •, Mn^{2+}; ▪, Mg^{2+}.

When the $(NH_4)_2SO_4$ precipitate of the pure enzyme was dissolved in the appropriate buffer and assayed immediately without a thiol-reducing agent, the activity was commonly only about 30% of that obtained in the presence of, e.g., 2–5 mM dithiothreitol. After a 2.5-h dialysis of the pure human prenyltransferase against 100 mM Tris-HCl buffer, pH 7.8, followed by another 1.5-h dialysis against a 10 mM buffer (to eliminate the inhibitory effects of the high ionic strength buffer), the enzyme became completely dependent on thiol-reducing agents for activity which was proportional to the logarithm of the thiol concentration up to a maximum activation (Fig. 4). Dithiothreitol gave half-maximal activation at 480 μM; dithioerythritol was almost as effective as dithiothreitol and gave half-maximal stimulation at 630 μM; cysteine, glutathione and 2-mercaptoethanol needed much higher concentrations than the dithiols to activate the enzyme (cf. Fig. 4).

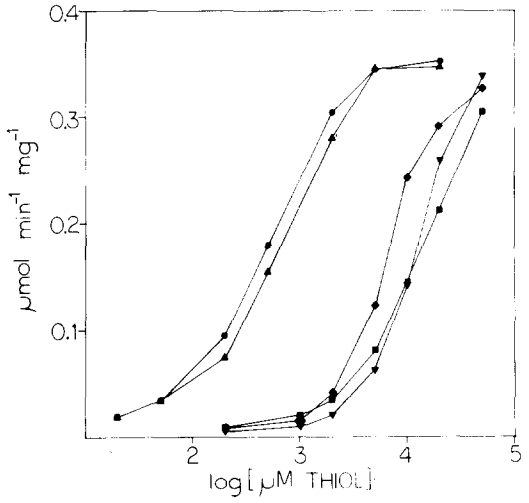

Fig. 4. Thiol requirements of human prenyltransferase. A sample of human prenyltransferase (300 μl, 15.5 μg/ml) was dialyzed for 2.5 h at 4°C against 250 ml of 100 mM Tris-HCl buffer, pH 7.8, to remove thiol-reducing agents and then for another 1.5 h against 10 mM Tris-HCl buffer, pH 7.8. Samples (5 μl) were assayed for 4 min in mixtures containing 0.50 μM substrates with various concentrations of thiol-reducing agents. Control incubations contained no thiols. Enzymic activity is shown as a function of the logarithm of thiol concentrations; •, dithiothreitol; ▲, dithioerythritol; ♦, L-cysteine; ▼, reduced glutathione; ■, 2-mercaptoethanol. Concentrations giving half-maximal activation were: 0.48 mM dithiothreitol; 0.63 mM dithioerythritol; 6.7 mM L-cysteine; 12.0 mM glutathione; 13.9 mM 2-mercaptoethanol.

The purified human liver prenyltransferase is quite stable when kept as a precipitate in neutral $(NH_4)_2SO_4$ solution at 60% saturation and 4°C. Even after 1 year of storage 30–50% of the original activity may remain. Solutions of the enzyme in 10 mM Tris-HCl buffer, pH 7.2–7.8, without any additives, are also stable at 4°C; we encountered no more than 5% losses of activity in 24 h.

Kinetic studies. Double-reciprocal plots of initial velocity data with the concentration of one substrate being varied and that of the other held constant are shown in Fig. 5. At the concentrations tested, 0.2–1.0 μM for isopentenyl pyrophosphate and 0.25–2 μM for geranyl pyrophosphate, the plots of $1/v$ against $1/[S]$ gave straight lines which intersected at points to the left of the Y axis and somewhat below the abscissae. The data are compatible with an ordered-sequential reaction mechanism as previously deduced for the pig liver enzyme [13] and the chicken liver enzyme [20].

The kinetic constants for the reaction catalyzed by the human prenyltransferase were evaluated from secondary plots of the data shown in Fig. 5. The rate equation for an 'ordered-sequential bi-bi' reaction

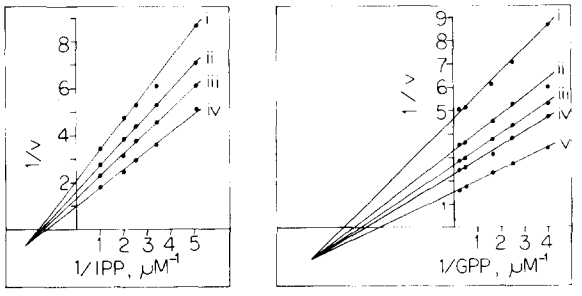

Fig. 5. Kinetics of human prenyltransferase at low substrate concentrations. Plots of $1/v$, $\mu mol^{-1} \cdot min \cdot mg$, against the reciprocal of the concentrations (μM^{-1}) of the varied substrate at different fixed concentrations of the second substrate are shown. Each point is the mean of four determinations. Figure on left: varied concentrations of isopentenyl pyrophosphate (IPP) at different fixed concentrations (μM) of geranyl pyrophosphate: (i) 0.25; (ii) 0.40; (iii) 0.60; (iv) 2.0 μM. On the right: varied geranyl pyrophosphate (GPP) concentrations at different fixed concentrations (μM) of isopentenyl pyrophosphate: (i) 0.20; (ii) 0.30; (iii) 0.40; (iv) 0.5; (v) 1.0 μM.

mechanism [21,22] written in the reciprocal form is:

$$\frac{1}{v} = \frac{1}{V_1}\left(\frac{K_s K_b}{[S_1][S_2]} + \frac{K_a}{[S_1]} + \frac{K_b}{[S_2]} + 1\right) \quad (1)$$

where v is the observed initial reaction rate, V_1 is the maximum reaction velocity in the forward direction, $[S_1]$ and $[S_2]$ are the concentrations of the substrates binding sequentially to the enzyme, geranyl pyrophosphate and isopentenyl pyrophosphate, respectively, K_s is the dissociation constant of the first substrate (or Cleland's 'inhibition constant', K_{ia} [22]) and is equal to the ratio of the rate constants, k_{-1}/k_{+1}, of the first reaction $E + S_1 \rightleftharpoons ES_1$, and K_a and K_b are the Michaelis constants for S_1 and S_2.

It can be shown [23] that by replotting the slopes and Y intercepts of the lines of Fig. 5 against the reciprocals of the concentrations of the substrate held constant, all the kinetic parameters can be evaluated provided such plots give straight lines. From three sets of experiments, similar to those shown in Fig. 5, and from the secondary plots of the slopes and intercepts of the lines, all of which were linear, we calculated the kinetic constants (with their standard deviations) for the human prenyltransferase. These were: K_a (geranyl pyrophosphate), 0.44 ± 0.07 μM, K_b (isopentenyl pyrophosphate), 0.94 ± 0.27 μM; K_s (from secondary plots of data when geranyl pyrophosphate was the varied substrate at several fixed concentrations of isopentenyl pyrophosphate), 0.14 ± 0.03 μM; the values for V_1 (V) were very close whether derived from the data when geranyl or isopentenyl pyrophosphate was the varied substrate, 1.07 ± 0.19 μmol · min^{-1} · mg^{-1} and 1.10 ± 0.23 μmol · min^{-1} · mg^{-1}, respectively.

The points of intersections of the lines of the double-reciprocal plots shown in Fig. 5 are below the abscissae, indicating that the binding of the constant substrate increased the apparent K_m value of the varied substrate [23]. This effect was more pronounced with isopentenyl pyrophosphate than with geranyl pyrophosphate as the constant substrate (Fig. 5 right). When the experiments were repeated with much higher concentrations of substrates, isopentenyl pyrophosphate as the varied substrate gave a very pronounced inhibition above 2 μM, and the replot of the data with geranyl pyrophosphate as the varied substrate showed that the nature of the inhibition was competitive (Fig. 6). No significant substrate inhibition was observed with geranyl pyrophosphate at the maximum concentration tested, 16 μM, a 17-fold excess over the K_m value for isopentenyl pyrophosphate.

The kinetics of human prenyltransferase were also studied in the absence of a thiol-reducing agent immediately after the $(NH_4)_2SO_4$ precipitate of the enzyme was dissolved in 10 mM Tris-HCl buffer, pH 7.8, which contained only traces of thiol. Such enzyme preparations, as mentioned earlier, showed in standard assays without thiol an activity only about 30% of that observed in the presence of 2 mM dithiothreitol. Fig. 7 illustrates the plots of the reciprocal of the initial reaction rates against that of the varied substrate at a single concentration of the fixed substrate, 0.50 μM, with and without 2 mM dithiothreitol. With geranyl pyrophosphate as the varied substrate a quasi competitive pattern was seen, suggesting that the change caused by the thiol-reducing agent facilitated the binding of geranyl pyrophosphate to the enzyme. When isopentenyl pyrophosphate was the varied substrate, the Lineweaver-Burk plots of the data obtained in the presence and absence of the dithiol gave a noncompetitive pattern (Fig. 7) consistent with the conclusion that the change caused by dithiothreitol affected only the binding of geranyl pyrophosphate and not that of isopentenyl pyrophosphate. The apparent K_m value

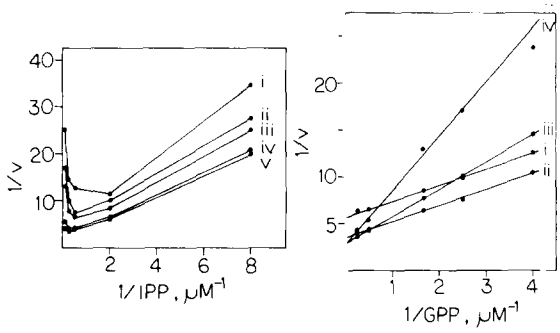

Fig. 6. Kinetics of human prenyltransferase at high concentrations of isopentenyl pyrophosphate. Figure at left shows plots of $1/v$, μmol^{-1} · min · mg, against the reciprocal of varied concentrations of isopentenyl pyrophosphate (1/IPP, μM^{-1}) at different fixed concentrations, μM, of geranyl pyrophosphate: (i) 0.25; (ii) 0.40; (iii) 0.60; (iv) 2.0; (v) 4.0 μM. Figure at right: similar plots as above with varied concentrations of geranyl pyrophosphate (1/GPP, μM^{-1}) at different fixed concentrations, μM, of isopentenyl pyrophosphate: (i) 0.50; (ii) 2.0; (iii) 4.0; (iv) 8.0 μM.

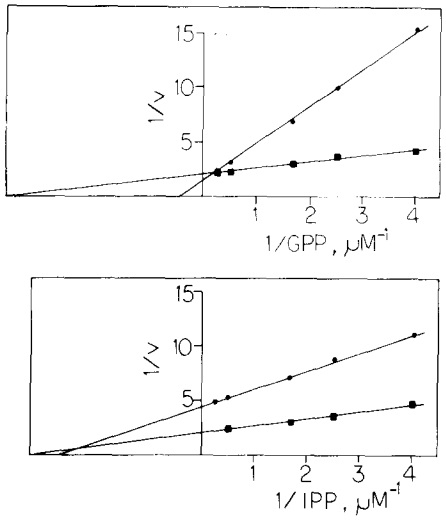

Fig. 7. Kinetics of human prenyltransferase with and without dithiothreitol. Samples of purified prenyltransferase, 7.5 ng in 20 μl, were assayed for 10 min in 0.5 ml standard incubations with [1-^{14}C] isopentenyl pyrophosphate of 56 Ci/mol specific activity either without, •, or with, ▪, 2 mM dithiothreitol. Upper graph: Lineweaver-Burk plots with varied concentrations of geranyl pyrophosphate (1/GPP, μM^{-1}) and with isopentenyl pyrophosphate at 0.50 μM. Lower graph: similar experiment to the one above, but with varied concentrations of isopentenyl pyrophosphate at a fixed concentration, 0.50 μM, of geranyl pyrophosphate.

for geranyl pyrophosphate in the absence of dithiothreitol was 2.15 ± 0.07 μM, whereas in its presence it was 0.26 ± 0.02 μM. In contrast, the apparent K_m values for isopentenyl pyrophosphate were the same within experimental error whether the dithiol was present or not, 0.35 ± 0.04 μM.

Inactivation of human prenyltransferase by phenylglyoxal and iodoacetic acid. We have reported recently on the inactivation of pig liver prenyltransferase by phenylglyoxal [3]. The human enzyme also reacts with this arginine-modifying reagent, but appears to be much more sensitive to it than the porcine enzyme. Thus, its inactivation by 10 mM phenylglyoxal was so rapid that it was not possible to carry out precise time experiments with such a concentration of the reagent. Instead, inactivation with 2 mM phenylglyoxal was followed for up to 60 min in 10 mM borate buffer at pH 8.2. The inactivation followed a pseudo-first-order kinetics with a single half-life of 20 ± 2 min (five determinations) in contrast to the biphasic inactivation of the pig liver enzyme [3]. Mg^{2+} (1 mM), which powerfully protected the porcine enzyme against inactivation by phenylglyoxal [3], had only a slight effect with the human enzyme and only increased the time of half-inactivation from 20 to 30 min. 3,3-Dimethylallyl and geranyl pyrophosphate, even in the absence of a divalent metal ion, both at 1 mM concentration, were the most effective antagonists to the inactivation by phenylglyoxal; the former increased the time for half-inactivation from 20 to 50 min, and in the presence of the latter, even after 60-min incubation with the phenylglyoxal, 70% of the enzymic activity remained. Thus the observations are compatible with our previous suggestion [3] that arginine residues are probably involved in the binding of the substrates to the enzyme.

Having seen the dependence of the human prenyltransferase on thiol-reducing agents for activity, we explored the effects of alkylation with iodoacetic acid in the presence and absence of 2 mM dithiothreitol or dithioerythritol. Since the most likely effect of the thiols was the reduction of a disulfide bond, we thought that the thiol-activated enzyme would be particularly sensitive to alkylation with iodoacetic acid. This expectation was not borne out by experiment. In 15-min incubations with 10 mM iodoacetic acid, at pH 7.3, in the absence of a dithiol 80–90% of the enzyme was inactivated, but in the presence of 2 mM dithiothreitol or dithioerythritol only 10–15% inactivation was seen. Even with 50 mM iodoacetic acid, which inactivated the enzyme in 15 min almost completely, with 2 mM of either dithiol in the incubations the extent of the inactivation was only 25–27%.

Immunological studies. The immunological examination of the human prenyltransferase arose from our study of the two forms of the pig liver enzyme referred to earlier. These immunological experiments with the porcine enzyme have not been reported previously. To investigate similarities and differences between the A- and B-forms of the porcine enzyme, we raised in rabbits antibodies to the B-form of the pig liver enzyme. The antiserum, when tested in double-immunodiffusion experiments, gave a sharp precipitin line with the pure B-form of the antigen. It also gave a sharp precipitin line with the A-form of the enzyme, but the line intersected the

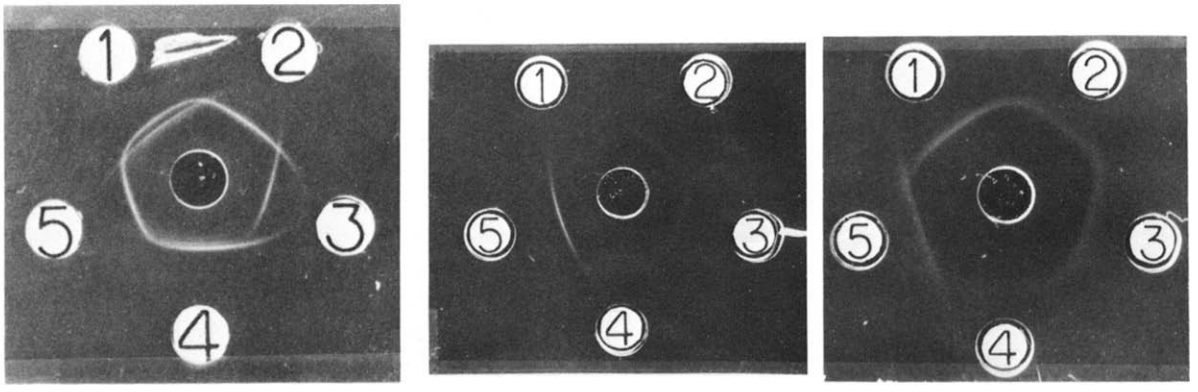

Fig. 8. Double-immunodiffusion plates with pig liver and human liver prenyltransferases. The center wells contained, from left to right, 8, 16, and 40 μl rabbit antiserum raised against the B-form of pig liver prenyltransferase. Left plate: well No. 1, 10 μl crude pig liver extract; wells 2 and 5, 1.8 μg pure porcine prenyltransferase, form-B; well No. 3, about 1 μg purified porcine prenyltransferase, form-A; well No. 4, partially purified B-form of porcine transferase incompletely separated from form-A. Center plate: wells 1–4 contain 4, 2, 1, and 0.5 μg purified human prenyltransferase; well No. 5, 1 μg pure form-B pig liver enzyme. Right plate: wells 1–4 contain 1.5, 1.0, 1.0 and 0.5 μg pure human prenyltransferase; well No. 5, 1.0 μg pure form-B pig liver enzyme.

precipitin line of the B-enzyme (Fig. 8, left plate). Crude extracts of the pig liver gave two precipitin lines, one identical with that given by enzyme B and the other identical with enzyme A.

The antiserum raised against the porcine enzyme showed in Ouchterlony plates cross-reactivity with the human enzyme also. However, the optimum conditions for precipitin-line formation with the porcine enzyme gave only weak lines with the human enzyme (Fig. 8, center plate), nonetheless sufficient to see spurs of nonidentity with the porcine B-form of the enzyme. By increasing the amount of antiserum in the center well (by repeated additions of 10 μl) stronger precipitin lines were formed with the human enzyme, but the porcine enzyme gave now a diffuse band, probably on account of antibody excess in the center well (Fig. 8, right plate).

In immunotitrations of fixed amounts of either the B-form of the porcine or the human enzyme with increasing volumes of the antiserum, the precipitation of both enzymes was linearly proportional to the volume of antiserum up to about 80% inactivation of each enzyme. By extrapolation of the titration curves to 100% inactivations we found that 10 μl of the antiserum were equivalent to 413 ng of the porcine enzyme and to 196 ng of the human enzyme (Fig. 9).

The antigen-antibody complexes formed with the human and porcine transferases were separately resuspended in 10 mM Tris-HCl buffer, pH 7.8, and 5 mM 2-mercaptoethanol and assayed for enzyme activity. Each had 15 ± 3% of the activity before

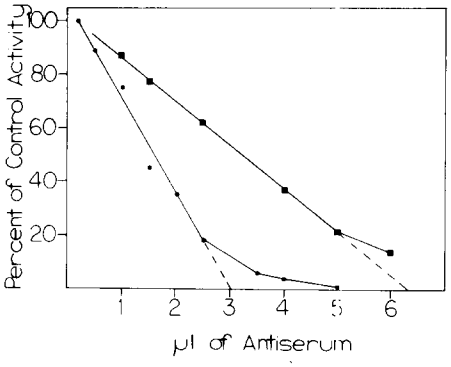

Fig. 9. Immunotitration of pig-liver and human prenyltransferase with rabbit antiserum against the B-form of the porcine enzyme. A total of 10 μl rabbit serum containing 0–10 μl antiserum and 10–0 μl control (preimmune) serum was added to 115 μl of 10 mM Tris-HCl buffer, pH 7.8/5 mM 2-mercaptoethanol in the 175 μl tubes of the Beckman Airfuge. The prenyltransferases (126 ng porcine enzyme, •, or 124 ng human enzyme, ▪) were added to the tubes in 10 μl of 1 mM dithiothreitol. The samples were mixed and kept at 4°C for 30 min, and then centrifuged at 132 000 × g for 20 min. The supernatants were assayed for prenyltransferase activity and were expressed as a percentage of the control value obtained with 10 μl of the preimmune serum.

precipitation, demonstrating that the antigen-antibody complexes retained some activity provided that the complexes did not dissociate upon resuspension in the buffer.

Discussion

The human liver prenyltransferase, the first of the sterol-synthesizing enzymes to be purified from a human tissue, shares several properties with the two best characterized prenyltransferases, the one isolated from pig liver and the other from chicken liver, but has distinguishing features of its own. It is a dimer of two indistinguishable subunits with a molecular weight of 74 000, somewhat less than that of the other two liver prenyltransferases. Its amino acid composition is very similar to that of the porcine enzyme with which it also shares common antigenic determinants. Unlike the porcine enzyme, which exists in two interconvertible forms, only one form of the human prenyltransferase could be observed under conditions which resolved the porcine enzyme into two distinct forms. The human enzyme has an absolute requirement for either of two divalent metal ions, Mn^{2+} or Mg^{2+}, but it has a far greater affinity for the former than for the latter, Mn^{2+} achieving a half-maximal activation at 3.7 μM as compared to 89.0 μM by Mg^{2+}. However, at saturating concentrations both ions give equal maximal activity. This behavior contrasts with that of the porcine enzyme which attains its maximum activity with 1 mM Mg^{2+}, but at the saturating concentration of 0.1 mM Mn^{2+} it has only about 30% of the maximum activity [13]. A peculiarity of the human enzyme, not recorded with other liver prenyltransferases, is its sensitivity to buffers of high ionic strength, which severely inhibit its activity.

Perhaps the most unique property of the human prenyltransferase is its complete dependence on thiols for activity after purification and dialysis against a Tris-HCl buffer, pH 7.8. This activation is rapid (instantaneous?) and occurs during the brief 1 or 2 min needed for setting up the incubations. The kinetic experiments with the partially active enzyme (cf. Fig. 7) showed that the activation by dithiothreitol (and presumably by the other thiols also) facilitated the binding of the allylic substrate to the enzyme. From this observation and the fact that the allylic pyrophosphate substrates protected the enzyme against inactivation by phenylglyoxal, we conclude that not only arginine residue(s) but also a cysteine residue is located at the active site, or at least at the allylic binding site of transferase. Inhibition of the pig liver prenyltransferase by iodoacetamide (2 mM), *N*-ethylmaleimide (5 μM) and *p*-hydroxymercuribenzoate (0.5 μM) and the reversal of inhibition caused by the latter agent by 1 mM 2-mercaptoethanol have been documented some time ago [13] and the participation of an SH-group in catalysis by this enzyme postulated.

In spite of the strong substrate inhibition observed in kinetic experiments at increasing fixed concentrations of isopentenyl pyrophosphate against varied concentrations of geranyl pyrophosphate, the kinetic data are still compatible with an ordered-sequential reaction mechanism catalyzed by the human transferase as was deduced from product inhibition studies for the pig liver enzyme [13]. The kinetics observed at high concentrations of isopentenyl pyrophosphate (cf. Fig. 6) indicate that this substrate can also bind to the binding site of the allylic substrate. Similar conclusions have been reached previously for the chicken liver enzyme [4,20], but the substrate inhibition became apparent in that case at lower concentrations of isopentenyl pyrophosphate than seen with the human enzyme. The kinetic behavior of the human as well as the chicken liver prenyltransferase is a good example of what Segel [24] described as "substrate inhibition in an ordered bireactant system" in which S_2, with structural similarities to S_1, may bind to the free enzyme, E, to yield a dead-end ES_2 complex. Segel [24] shows that S_2 at high fixed concentrations becomes a competitive inhibitor of S_1 when the concentrations of the latter are varied and that at very high fixed concentrations of S_2 the slope of the $1/v$ vs. $1/[S_1]$ plots is given by Eqn. 2 (Segel's equation IX-36)

$$\text{slope}_{1/S_1} = \frac{K_a}{V_1 K_i}[S_2] + \frac{K_s K_b}{V_1}[S_2]^{-1} + \frac{K_a}{V_1} \cdot \left(1 + \frac{K_s K_b}{K_a K_i}\right) \quad (2)$$

where K_i is the dissociation constant of the ES_2 complex, and the other symbols are those defined for

Eqn. 1. By using this equation and the slopes of $1/v$ vs. $1/[S_1]$ plots obtained at the two highest concentrations of S_2, 4 and 8 μM, (cf. Fig. 6) and the values of the other kinetic parameters, we obtained values of 0.67 and 0.70 μM for K_i. These values are also commensurate with the data obtained at concentrations of isopentenyl pyrophosphate below 2 μM (cf. Fig. 5 right), which indicated that concentrations of S_2 increasing even from 0.2 to 1 μM increased the apparent K_m values of geranyl pyrophosphate.

We have inferred previously [3,7] that the two forms of the pig liver prenyltransferase represent not only two different oxidation-reduction states of the same protein, but that they may also have different conformations as evidenced by the greater negative surface charge of form-B than that of form-A and by the anomalous behavior of form-A in the ultracentrifuge. The cross-reactivity, but immunologic non-identity of form-A with form-B in double immunodiffusion experiments (Fig. 8, left plate) is explicable by the assumption that the diffusion coefficient of form-A was larger than that of form B. The cross-reactivity of the human transferase with antibodies raised against the porcine enzyme suggests substantial homology between the two enzymes which is supported also by their almost identical amino acid composition.

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