Photoaffinity Labeling of the Catalytic Site of Prenyltransferase†

David N. Brems and Hans C. Rilling*

ABSTRACT: Three photoreactive substrate analogues, *o*-azidophenethyl pyrophosphate, *p*-azidophenethyl pyrophosphate, and 3-azido-1-butyl pyrophosphate, have been synthesized as site-directed probes to label the catalytic site of prenyltransferase. Due to the relatively poor affinity of *p*-azidophenethyl pyrophosphate and 3-azido-1-butyl pyrophosphate for the enzyme, only *o*-azidophenethyl pyrophosphate (aryl azide) was utilized for photoaffinity labeling. This aryl azide has a UV absorption maximum at 250 nm. In the absence of activating light, binding studies demonstrate that the *o*-aryl azide competes for binding with both the natural substrates, isopentenyl pyrophosphate and geranyl pyrophosphate. More than 90% enzymatic activity is lost when enzyme is irradiated in the presence of the aryl azide as compared to irradiation in the absence of the azide, and the protein loses its capacity for substrate binding in direct proportion to photolabeling. A stoichiometry of 2 mol of affinity label covalently bound per mol of enzyme dimer was established with [1-^{3}H]-*o*-azidophenethyl pyrophosphate. Since there are two catalytic sites per enzyme dimer, the *o*-aryl azide appears specifically to label the enzyme at its catalytic sites. Additional evidence that the reagent was specific for the catalytic site came from the observation that farnesyl pyrophosphate afforded complete protection against photoinactivation, while isopentenyl pyrophosphate provided partial protection. Gel isoelectric focusing verified this stoichiometry and indicated that the labeled enzyme has a more acidic isoelectric point than the native enzyme.

Prenyltransferase (EC 2.5.1.1) catalyzes the head-to-tail condensation between an allylic isoprenyl pyrophosphate and isopentenyl pyrophosphate. This condensation is the fundamental chain elongation reaction of terpene biosynthesis and leads to the formation of many diverse products such as sterols, carotenes, dolichols, and hydrocarbon side chains of the respiratory coenzymes. Prenyltransferase (farnesyl pyrophosphate synthetase) has been purified to homogeneity from several sources (Eberhardt & Rilling, 1975; Reed & Rilling, 1975; Dorsey et al., 1966).

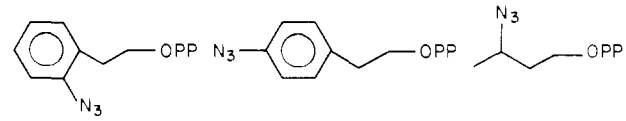
Studies from this laboratory and that of C. Dale Poulter (Department of Chemistry, University of Utah) have shown that the reaction proceeds by an ionization–condensation–elimination mechanism which is initiated by the formation of a carbonium ion (or an ion pair) from the allylic substrate (Poulter & Rilling, 1976, 1978; Poulter & Satterwhite, 1977; Poulter et al., 1977; Brems & Rilling, 1977; Rilling, 1979). The involvement of a carbonium ion in enzymatic reactions is rare, with possibly lysozyme (Jenks, 1969) and fumarase (Mildvan, 1974) being the only precedents. Because of the unusual nature of this reaction and because the mechanism may be general to other prenyl transfer reactions, we have decided to investigate the sequence of the peptides in the catalytic site. Hopefully, this will provide some insight as to how the reaction is catalyzed.

Since prenyltransferase does not make covalent linkages with its substrates and since there is no clue as to the identity of the amino acids in the catalytic site, photoaffinity labels provide the most direct approach to labeling the catalytic site of this enzyme. In this paper we report the photoaffinity labeling of the catalytic site of prenyltransferase.

Materials and Methods

Preparation of Photolabile Substrate Analogues I, II, and III

o-Azidophenethanol. *o*-Aminophenethanol was purchased from Aldrich Chemical Co. The corresponding *o*-azidophenethanol was prepared by the procedure of Smith & Brown (1951). Spectral data: IR ν_{max} 3350, 2100, and 775 cm^{-1}; ^{1}H NMR ($CDCl_3$) 7.2 (4 H, m), 3.8 (2 H, m, J = 6 Hz), 2.8 (2 H, t, J = 6 Hz), 1.5 (1 H, s); E_{250} = 2000 L cm^{-1} mol^{-1} in ethanol.



I II III

[1-^{3}H]-o-Azidophenethanol. One gram of *o*-aminophenethanol was reacted neat with 1 equiv of acetic anhydride. After 0.5 h, the solution solidified. The product was recrystallized from absolute ethanol with a yield of 95%. The crystalline solid has a melting point of 103 °C. Spectral data: ^{1}H NMR ($CDCl_3$) 8.8 (1 H, broad s), 7.2 (4 H, m), 3.9 (2 H, m, J = 5 Hz), 2.8 (2 H, t, J = 5 Hz), 2.4 (1 H, s), 2.1 (3 H, s). The aldehyde was then formed by oxidation with 3 equiv of chromium trioxide in water which was added to *N*-acetyl-*o*-aminophenethanol in pyridine. The reaction was monitored and products were detected by fluorescence quenching on silica gel thin-layer chromatography. A mixture of at least three products was obtained.

One hundred milligrams of this mixture was chromatographed on preparative plates of silica gel G with diethyl ether as solvent. The 75 mg of *N*-acetyl-*o*-aminophenylacetaldehyde (R_f 0.5) thus obtained was reduced to [1-^{3}H]-*N*-acetyl-*o*-aminophenethanol by [^{3}H]$NaBH_4$. This material was then hydrolyzed with 6 N HCl at 100 °C for 1 h. Excess HCl was then removed under vacuum, and the product was neutralized with NH_4OH. After excess NH_4OH was removed under vacuum, [1-^{3}H]-*o*-aminophenethanol was dissolved in ethyl ether and the borates were removed by filtration. The pyrophosphate ester of [1-^{3}H]-*o*-azidophenethanol was prepared as described below. The radioactive pyrophosphate (sp act. 31 μCi/μmol) has an identical R_f value in 2-propanol–ammonia–water (6:3:1) and extinction coefficient as I.

p-Azidophenethanol. *p*-Aminophenylacetic acid was purchased from Aldrich Chemical Co. and was reduced to the corresponding alcohol with $LiAlH_4$. Spectral data: IR ν_{max} 3150, 1050, and 835 cm^{-1}; ^{1}H NMR ($CDCl_3$) 6.9 (2 H, d, J = 7 Hz), 6.5 (2 H, d, J = 7 Hz), 3.7 (4 H, m, J = 5 Hz), 2.7 (2 H, t, J = 5 Hz), 1.5 (1 H, s). The remaining synthesis was identical with that for I.

† From the Department of Biochemistry, University of Utah College of Medicine, Salt Lake City, Utah 84132. *Received August 30, 1978; revised manuscript received December 12, 1978.* This research was supported by National Institutes of Health Grant AM-13140.

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3-Azido-1-butanol. 1,3-Butanediol was purchased from Aldrich Chemical Co. One gram of the diol was reacted with 1 equiv of acetic anhydride in 8 equiv of pyridine for 24 h. After the excess pyridine was neutralized with HCl, the products were extracted into ethyl ether. The primary acetylated alcohol was separated from other products by chromatography on a Woelm (ICN) silica gel column, with 30% ethyl acetate in hexane as solvent. A yield of 50% was obtained. Spectral data: IR ν_{max} 3400, 1740, 1240, and 1050 cm^{-1}; ^1H NMR ($CDCl_3$) 4.4–3.7 (3 H, m, J = 6 Hz), 2.25 (1 H, s), 2.0 (3 H, s), 1.7 (2 H, q, J = 6 Hz), 1.2 (3 H, d, J = 6 Hz). 1-Acetoxy-3-hydroxybutanol was reacted at reflux with 1.5 equiv of carbon tetrabromide and 1 equiv of triphenylphosphine in methylene chloride. 1-Acetoxy-3-bromobutanol (70%) was isolated by distillation. Spectral data: IR ν_{max} 1745, 1240, 1050, and 670 cm^{-1}. 1-Acetoxy-3-bromobutanol was hydrolyzed with 1 equiv of Na_2CO_3 in water–methanol. 3-Bromo-1-butanol (30%) was resolved from other products on a silica gel column (solvent system 10% ethyl acetate in hexane). Spectral data: ^1H NMR ($CDCl_3$) 4.3 (1 H, m, J = 6 Hz), 3.8 (2 H, t, J = 6 Hz), 2.2 (1 H, s), 2.0 (2 H, q, J = 6 Hz), 1.7 (3 H, d, J = 6 Hz); IR ν_{max} 3400 cm^{-1}. 3-Bromo-1-butanol was refluxed for 10 h with a 10-fold excess of potassium azide in acetonitrile and a catalytic amount of 18-crown-6 ether. 3-Azido-1-butanol (98%) was used without further purification. Spectral data: IR ν_{max} 3400, 2100, 1250, 1060 cm^{-1}; ^1H NMR ($CDCl_3$) 3.7 (3 H, m, J = 6 Hz), 2.2 (1 H, s), 1.7 (2 H, q, J = 6 Hz), 1.3 (3 H, d, J = 6 Hz); mass spectrum m/e 116 (M^+) 113, 88, 73, 70 (base).

The pyrosphosphate esters of the above alcohols were prepared by the method of Cramer as described by Cornforth & Popjak (1969) and purified by ion-exchange chromatography on Dowex AG-1-X8 formate from Bio-Rad by use of a linear ammonium formate gradient in methanol (Sofer & Rilling, 1969). Purity of the pyrophosphates was verified by thin-layer chromatography on ammonium sulfate impregnated silica gel H plates with $CHCl_3$–CH_3OH–H_2O (5:5:1) as solvent or on Whatman No. 1 paper with 2-propanol–ammonia–water (6:3:1) as solvent.

Concentrations of I, II, and III were determined by analysis of total phosphate as described by Reed & Rilling (1976). Presence of the azide after phosphorylation was confirmed by applying I to a thin-layer plate of silica gel H containing fluorescent indicator. After irradiation of several of the spots by UV light, the plate was developed with 2-propanol–ammonia–water (6:3:1). The plate was then visualized under UV light. If I had not been irradiated prior to chromatography, a single spot of R_f value 0.23 was obtained. If I had been irradiated, the majority of the material remained at the origin.

Preparation of Enzyme

The enzyme was purified from avian liver by the method of Reed & Rilling (1975). The crystals were stored at 4 °C as a suspension in buffered ammonium sulfate solution containing dithiothreitol. Enzyme concentrations were determined by absorbance at 280 nm, by utilization of an extinction coefficient of 88 400 L mol^{-1} cm^{-1}. Enzyme activity was determined as before (Reed & Rilling, 1975).

Photoaffinity Labeling

The protein was desalted on a Sephadex G-25 column (V_0 = 7.2 mL), equilibrated with 50 mM potassium Tes[1] buffer, pH 7.0, containing 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 100 mM KCl. Dithiothreitol which is usually included to stabilize the enzyme was omitted since aryl azides are reduced to the corresponding amines by this reagent (Staros et al., 1978). Solutions of enzyme and analogue were photolyzed at room temperature in a 1-mm path-length quartz cuvette situated on the surface of a Mineralight UVS-11 (maximum emission 254 nm).

The extent of photoaffinity labeling was monitored by loss of enzyme activity. The fractional photoinactivation of enzyme was calculated by the following expression

[(act. of enzyme irradiated in absence of affinity label) – (act. of enzyme irradiated in presence of affinity label)]/ (act. of enzyme irradiated in absence of affinity label)

One-minute irradiation of enzyme in the absence of I resulted in 5–10% loss of enzymatic activity, depending on enzyme concentration. Each sequential 1-min irradiation gave an additional but lesser (1–5%) loss of enzyme activity. The loss of activity was consistent for a given protein concentration.

Substrate Binding Studies

Substrate binding was measured at 4 °C using forced equilibrium dialysis (Reed & Rilling, 1976).

Radioisotope Determination

[1-^{3}H]-*o*-Azidophenethyl pyrophosphate was 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 (New England Nuclear). Products of enzyme assays (hexane–soluble acid hydrolysis products) were dissolved in 10 mL of toluene containing 0.4% Omnifluor. Radioactivity was determined by liquid scintillation spectrometry.

Substrates

[1-^{3}H]Geranyl pyrophosphate was prepared as described by Cornforth & Popjak (1969). The pyrophosphate esters were purified by ion-exchange chromatography (Sofer & Rilling, 1969). [1-^{14}C]Isopentenyl pyrophosphate was purchased from Amersham/Searle.

Electrofocusing

Gel isoelectric focusing in 7.5% polyacrylamide gels and pH 5–7 range ampholine was according to Wrigley (1971).

Results

Inhibition Constants of I, II, and III. Kinetically obtained inhibition constants for I are 18 and 9 μM, for II are 60 and 85 μM, and for III are 125 and 60 μM against geranyl and isopentenyl pyrophosphate, respectively. K_m values for natural substrates isopentenyl pyrophosphate and geranyl pyrophosphate for the synthesis of farnesyl pyrophosphate are both 0.10 μM (Poulter & Laskoviks, unpublished results). Since the kinetic data suggest that I binds 5–10 times more tightly than II or III, only I was used in the following experiments.

Half-Life of Photodecomposition. I has a UV absorption maximum at 250 nm with an extinction coefficient of 2230 L mol^{-1} cm^{-1} in water. The half-life for photolytic formation of the nitrene from the corresponding azide was determined by loss of absorbance at 250 nm. A maximum loss of absorbance at 250 nm was obtained after 60 s, while the absorbance after irradiating for 30 s showed that one-half the azide had disappeared.

Photoaffinity Labeling of Prenyltransferase. The extent of photoaffinity labeling of prenyltransferase as a function of the concentration of I was monitored by loss of enzyme activity

[1] Abbreviations used: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate.

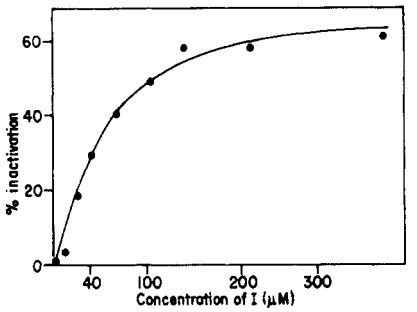

FIGURE 1: Effect of increasing concentrations of I on photodependent inactivation of prenyltransferase. Appropriate amounts of I were added to enzyme (2.51 μM) and photolyzed for 1 min. Aliquots were then removed for determination of enzyme activity. Irradiation of the enzyme in absence of I resulted in 10% loss of enzymatic activity.

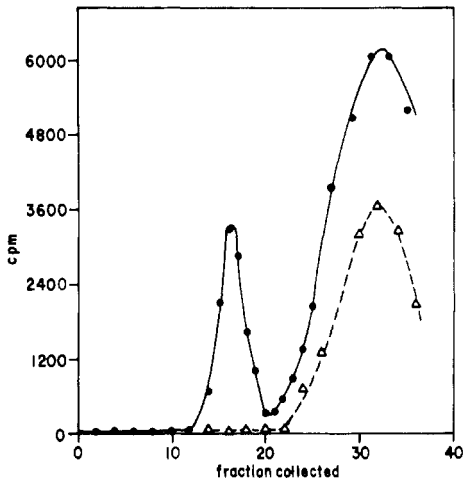

FIGURE 2: Incorporation of photoaffinity label covalently bound to prenyltransferase. Enzyme was labeled with [1-^{3}H]-*o*-azidophenethyl pyrophosphate to the extent of 91% loss of enzyme activity. Labeled enzyme (○) was then passed over Sephadex G-25 column; 0.5-mL fractions were collected and were then measured for radioactivity. (△) is the elution pattern of [1-^{3}H]-*o*-azidophenethyl pyrophosphate minus protein.

(Figure 1). The observed loss of activity was not due to competitive inhibition by the analogue since the final concentration of I in the assay mixture was less than one-hundredth of its K_i values. Also, in control experiments we found the photolysis product was a poorer inhibitor than its unphotolyzed counterpart. Irradiation of enzyme in the absence of I resulted in 10% loss of enzymatic activity.

In order to obtain more extensive photolabeling, the enzyme was irradiated with 30 mol of I/mol of enzyme for several 1-min intervals. After each irradiation, the amount of I was restored to the original concentration and the process repeated. The percent enzyme activity remaining after each of the five irradiations was 61, 34, 19, 9, and 6.

This experiment demonstrates that by cycling the enzyme and I through a series of photolytic reactions nearly complete inactivation of the enzyme is achieved. Control experiments showed that five 1-min irradiations of the enzyme in the absence of I resulted in a 10% loss of enzyme activity. In this control experiment, the principal loss of enzyme activity by irradiation occurred with the first exposure; subsequent irradiations caused smaller losses.

Stoichiometry of Photoaffinity Label Covalently Attached to Prenyltransferase. Our intent is to selectively label prenyltransferase at the catalytic site. Therefore, the stoichiometry of photoaffinity labeling is important. Obviously, nonspecific labeling would complicate sequencing of peptides from the

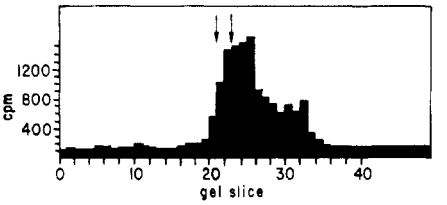

FIGURE 3: Enzyme was photoderivatized with [1-^{3}H]-*o*-azidophenethyl pyrophosphate to the extent of 90% loss of enzymatic activity and subjected to gel isoelectric focusing, pH 5–7. Native enzyme appeared as two bands indicated by the arrows. Two bands of protein observed on isoelectric focusing of pure proteins are observed occasionally (Hare et al., 1978). After photoderivatization, a broad, diffuse series of bands resulted. Gels were sliced into 2-mm segments and radioactivity was determined.

Table I: Stoichiometry of Photoaffinity Label Covalently Bound to Prenyltransferase[a]

% inactivation	19	32	54	66	70	78	87	93
mol of photoaffinity label bound/ mol of enzyme	0.1	0.3	0.4	0.8	1.0	1.2	1.5	2.2

[a] Prenyltransferase was photolyzed with varying concentrations of I to obtain the fractional inactivation as shown in the table. After photolysis, 0.3 mg of bovine serum albumin was added and protein was precipitated and washed with Cl_3AcOH. The radioactivity associated with protein was then determined.

catalytic site. Stoichiometry of labeling was determined by three different methods. Prenyltransferase was affinity labeled with [1-^{3}H]-*o*-azidophenethyl pyrophosphate to the extent of 91% loss of enzyme activity. The labeled enzyme was then passed through a Sephadex G-25 column. Two peaks of radioactivity were eluted, and the radioactivity found in the excluded fractions (13–19) when summed indicated that 2.1 mol of affinity label were bound per mol of enzyme dimer (Figure 2). In a second experiment, enzyme that had been photoaffinity labeled to 93% loss of enzymatic activity was precipitated with 10% Cl_3AcOH. The protein was collected by centrifugation and was suspended and washed successively with 5 and 1% Cl_3AcOH. The stoichiometry observed was 1.89 mol of photoaffinity label bound per mol of enzyme dimer. Control experiments indicated negligible binding without photolysis. In a third experiment, protein, photolyzed to the extent of 90% inactivation in the presence of [1-^{3}H]-*o*-azidophenethyl pyrophosphate, was subjected to gel isoelectric focusing in the pH range 5–7. A rather broad, diffuse series of bands was observed on staining. When gels were sliced into 2-mm segments, the radioactivity recovered corresponded directly to the protein-containing region (Figure 3). The radioactivity in the area containing greater than 300 cpm/slice was summed. A value of 2.07 mol of affinity label/mol of enzyme dimer was calculated from the resultant total of radioactivity and the amount of protein applied to the gel.

Enzyme, photoaffinity labeled to varying extents of loss of catalytic activity, was analyzed for radioactivity after acid precipitation. The results (Table I) show a rough correlation of loss of catalytic function with photoderivatization. However, since photolabeling lags loss of activity, it is possible that derivatization of one subunit results in loss of catalytic activity with retention of ability in the second subunit to bind substrates and substrate analogues.

Protection against Photoinactivation by Substrates and Product. The observed stoichiometry strongly suggests that the enzyme is only derivatized at its catalytic site. If this were the case, one would anticipate that either substrates or product would protect the enzyme against inactivation. Prenyltransferase was incubated with I and varying concentrations

Table II: Effect of Ligand Protection against Photoaffinity Labeling[a]

	concentration (μM)						
ligand	0	1	2	4	8	16	20
FPP	55	18	13	8	6	6	
GPP	51						51
IPP	54	47	43	38	35	27	

[a] Various concentrations of ligand were included during photoaffinity labeling (14.3 μM of I and 0.28 μM enzyme). Irradiations were for 1 min. Aliquots were then removed for catalytic activity determination. After dilution of aliquots from affinity-labeling reaction mixture into the assay mixture, controls showed that competitive inhibition by FPP, GPP, and IPP was negligible during enzyme assay. The data are expressed as percent of initial activity.

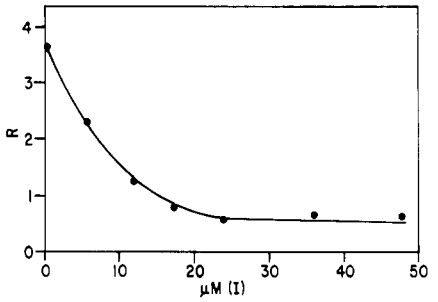

FIGURE 4: Competition between [1-^{14}C]isopentenyl pyrophosphate and I for binding to prenyltransferase in the presence of 1 mM $MgCl_2$. Enzyme (2.69 μM) was saturated with 20 μM isopentenyl pyrophosphate. R is the number of moles of [1-^{14}C]isopentenyl pyrophosphate bound per mol of enzyme.

of either geranyl, isopentenyl, or farnesyl pyrophosphate. The data, shown in Table II, demonstrate that farnesyl pyrophosphate afforded complete protection at 4–8 μM, isopentenyl pyrophosphate afforded about 50% protection at 16 μM, while geranyl pyrophosphate failed to protect the enzyme. The experiments show very clearly that I is destroying enzymatic activity by reacting with the catalytic site.

Since geranyl pyrophosphate did not protect this enzyme against photoinactivation by I, it is probable that the enzyme is being selectively derivatized in the homoallylic site. Binding studies also show that this analogue competes effectively with isopentenyl pyrophosphate for the enzyme, reducing its binding sevenfold at 20 μM concentration (Figure 4). On the other hand, binding of geranyl pyrophosphate was only reduced about twofold by the analogue at concentrations up to 50 μM (Figure 5). These results and the kinetic experiments suggested that I could be reacted selectively at the homoallylic site. Therefore, milligram quantities of the enzyme were photoderivatized to varying extents. Unbound I was removed by passing labeled enzyme over Sephadex G-25, and the binding of natural substrates was determined in the usual manner. Enzyme that had lost 85% activity due to photoaffinity labeling did not bind a measurable amount of isopentenyl pyrophosphate or geranyl pyrophosphate, while enzyme that was 36% inactivated by photoaffinity labeling bound 1.1 mol of isopentenyl pyrophosphate and 0.63 mol of geranyl pyrophosphate per mol of enzyme. The native enzyme binds 4 mol of isopentenyl or 2 mol of geranyl pyrophosphate; thus, partial inactivation of the enzyme reduces its ability to bind both substrates proportionally.

We then attempted to demonstrate that the enzyme, inactivated in the presence of geranyl pyrophosphate, would retain its ability to bind the allylic substrate. In this experiment, the enzyme was saturated with geranyl pyrophosphate and then subjected to photoaffinity labeling. After

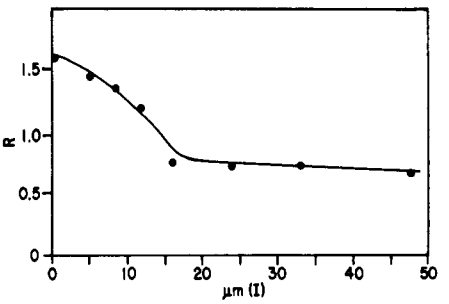

FIGURE 5: Competition between [1-^{3}H]geranyl pyrophosphate and I for binding to prenyltransferase in the presence of 1 mM $MgCl_2$. Enzyme (2.78 μM) was saturated with 25 μM geranyl pyrophosphate. R is the number of moles of [1-^{3}H]geranyl pyrophosphate bound per mol of enzyme.

removal of small molecules by gel filtration, binding of geranyl pyrophosphate and isopentenyl pyrophosphate was measured and found to be essentially the same as for enzyme whose allylic site had not been protected. Thus, protection of the allylic site was not possible.

Discussion

Prenyltransferase catalyzes the carbon-to-carbon bond-forming reaction which is the polymerizing step in polyprenol biosynthesis. This condensation proceeds by an ionization–condensation–elimination reaction, and it is thought that the reaction is initiated by the formation of a carbonium ion (or an ion pair) from the allylic substrate (Poulter & Rilling, 1976; Poulter & Satterwhite, 1977; Poulter et al., 1977; Brems & Rilling, 1977). Enzymatically catalyzed reactions that proceed by carbonium ions are unusual, and consequently knowledge of the structure of the peptides involved in the active site would be interesting and perhaps useful in further delineating the nature of the catalytic process. One way to identify the portion of enzyme responsible for catalysis would be to use photoreactive substrate analogues. This approach seemed quite reasonable since prenyltransferase has a fairly low specificity for the hydrocarbon portion of the substrate. For example, substitution of a methyl group for a hydrogen at either C(4) or C(5) of 3-methyl-2-butenyl or 3-methyl-3-butenyl pyrophosphate provided analogues that functioned as substrates (Popjak et al., 1969a,b; Ogura et al., 1970; Nishino et al., 1971a,b, 1973). Initially, we sought to prepare analogues of the 5-carbon substrates with the photolabile group attached at the 3 or 4 methyl. These would resemble the substrate analogues in which extra methyl groups had been placed in this position. We prepared 4-azido-3-methyl-2-butenol but found that it was unstable with a half-life of hours. Consequently, we prepared III hoping that it would interact strongly with the enzyme. However, its K_i value turned out to be high, indicating about a 1000-fold weaker interaction with the enzyme than its natural substrates. The preparation of the aryl azides I and II was suggested by the finding of Saito (1976), in Ogura's laboratory, that phenethyl pyrophosphate was a competitive inhibitor of prenyltransferase. In addition, I and II have the advantage of having absorption maximum at longer wavelengths than alkyl azides (Knowles, 1972), permitting photolabeling with less potential destruction of protein. Of these analogues, I has the lowest K_i values and thus was the reagent of choice for photolabeling experiments.

This aryl azide was quite effective in photolabeling prenyltransferase. When subjected to a single exposure of activating light, as much as 60% of the activity of prenyltransferase was lost if 200 μM of the analogue was included with the enzyme. In experiments in which the enzyme was repeatedly

exposed to UV light in the presence of I, the enzyme was nearly completely inactivated.

Acid precipitation, Sephadex G-25 chromatography, and gel isoelectric focusing of affinity-labeled enzyme show that the photoaffinity label is covalently bound to protein. In addition, precipitation of the affinity-labeled enzyme with acid indicates that the bond between affinity label and enzyme is not acid labile.

Selective derivatization of the protein is extremely important since nonspecific labeling would lead to misleading results on degradation of the protein. The following experiments indicate that this labeling was specific for the catalytic site of this enzyme. The loss of catalytic activity roughly paralleled the incorporation of covalently linked affinity label into the protein as well as the loss of the ability of the enzyme to bind its substrates as measured by forced equilibrium dialysis. Also, the stoichiometry of labeling which was determined by several methods indicated that 2 mol of analogue were bound per mol of enzyme which is, of course, one per catalytic site. In addition, the plateau obtained for inactivation as a function of analogue concentration indicates relatively little nonspecific labeling of the protein at high concentration of the arylazide.

The most convincing evidence for site-specific labeling comes from the finding that at low concentrations farnesyl pyrophosphate was able to protect the enzyme against photoinactivation. Other studies have shown that farnesyl pyrophosphate competes effectively with both allylic and homoallylic substrates and thus is envisioned as blocking the entire catalytic site (King & Rilling, 1977). When the individual substrates were tested for their ability to protect the enzyme, only isopentenyl pyrophosphate protected the enzyme and at that only partial protection was observed. These data, along with the K_i values established for the analogue, strongly suggest that the analogue interacts selectively at the homoallylic site. However, a comparison of the ability of partially inactivated enzyme to bind its individual substrates showed that the capacity to bind both substrates was lost in parallel. Also, when geranyl pyrophosphate was included during irradiation to protect the allylic portion of the catalytic site, forced equilibrium dialysis again showed that the protein again had lost its capacity to bind both substrates to an equal extent. Thus, the evidence clearly indicates that a catalytic site, once reacted with the photolabel, loses its capacity to bind both substrates as well as its catalytic function.

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