to fibrinogen in the same species. The availability of the new fluorescent substrates should facilitate a more rigorous examination of protein modifications of this type.

Acknowledgments

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Isolation and Characterization of a Photoaffinity-Labeled Peptide from the Catalytic Site of Prenyltransferase†

David N. Brems,‡ Eveline Bruenger, and Hans C. Rilling*

ABSTRACT: Previously we presented evidence for the selective modification of the catalytic site of prenyltransferase by photoaffinity labeling with *o*-azidophenylethyl pyrophosphate [Brems, D. N., & Rilling, H. C. (1979) *Biochemistry 18*, 860]. In the present work, we report the isolation and characterization of a CNBr fragment of 30 amino acid residues from the photoaffinity-labeled enzyme. This CNBr fragment contains over 80% of the total label attached to prenyltransferase as a result of photoaffinity labeling. Several lines of evidence indicate that a number of residues in this CNBr fragment have been modified. First, Edman degradation of this labeled peptide demonstrates that at least 16 of the 30 amino acids have been modified by the photoaffinity reagent. The two most extensively modified amino acids are a specific arginine and alanine. Second, two-dimensional chromatography of Pronase digestions of the labeled CNBr fragment indicates that at least 11 different products resulted from photoaffinity labeling. Third, peptide maps of a trypsin digest of this CNBr fragment show that the attached affinity label is distributed among at least three of the resulting products of tryptic hydrolysis. Finally, comparison of amino acid analysis of this CNBr fragment with that of its counterpart isolated from native enzyme is consistent with the modification of a number of amino acids rather than a few by the photoaffinity labeling process.

Prenyltransferase (farnesyl pyrophosphate synthetase, EC 2.5.1.1) is an enzyme of molecular weight 86 000. It consists of two identical subunits with one catalytic site per subunit. Prenyltransferase catalyzes two consecutive head-to-tail condensations between isopentenyl pyrophosphate and an allylic pyrophosphate.

The reaction catalyzed by prenyltransferase is unique since it is the first biochemical reaction unequivocally shown to proceed via a carbonium ion (Poulter & Rilling, 1976, 1978; Poulter & Satterwhite, 1977; Poulter et al., 1977; Brems & Rilling, 1977; Rilling, 1979). The mechanism of this reaction

† From the Department of Biochemistry, University of Utah College of Medicine, Salt Lake City, Utah 84108. *Received July 18, 1980; revised manuscript received February 12, 1981.* This investigation was supported by National Institutes of Health Grants AM 13140 and AM 21745.

‡ Present address: Department of Biochemistry, University of Iowa, Iowa City, IA 52242.

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has been exhaustively studied by kinetic and chemical methods, so the next logical step would be to determine the primary sequence of the catalytic site in hopes of identifying the amino acids involved in substrate binding and catalysis. To accomplish this, we have utilized photoaffinity labeling to derivatize the catalytic site of this enzyme. In 1979, we (Brems & Rilling, 1979) reported the synthesis of several photoreactive substrate analogues. One of these, *o*-azidophenylethyl pyrophosphate, has relatively good affinity for the enzyme, as determined by kinetic and binding experiments. We also found that more than 90% of the enzymatic activity is lost when the enzyme is irradiated in the presence of the photoaffinity reagent as compared to irradiation in the absence of the reagent. In addition, the protein loses its capacity for substrate binding in direct proportion to photolabeling. Further evidence that the reagent was specific for the catalytic site came from the observation that farnesyl pyrophosphate afforded complete protection against photoinactivation. A stoichiometry of 2 mol of affinity label covalently bound per mol of enzyme dimer was established with *o*-azidophenyl[1-^{3}H]ethyl pyrophosphate. Since there are two catalytic sites per enzyme dimer, the *o*-aryl azide appears specifically to label the enzyme at its catalytic site. In the present paper, we extend this study and report the isolation and characterization of peptides that have been modified by this site-directed probe.

Materials and Methods

Enzyme Preparation. The purification procedure of Reed & Rilling (1975) was altered by including a $CaCl_2$ precipitation step in order to reduce the lipid content. Under the original procedure, the high lipid content of chicken liver, usually available, resulted in poor separations in the first centrifugation and the first ammonioum sulfate precipitation.

Typically, 4 kg of liver was thawed in 9.4 L of 50 mM imidazole buffer, pH 7.0, containing 10 mM 2-mercaptoethanol and homogenized in a Waring blender for 1 min. $CaCl_2$ was added to a final concentration of 40 mM, and the solution was centrifuged at 10 000 rpm for 30 min. The supernate was decanted through eight layers of cheesecloth and adjusted to 40 mM phosphate, pH 7.0. The ammonium sulfate precipitations were as before, while buffer for dialysis was 5 mM potassium phosphate, pH 7.0, containing 1 mM EDTA[1] and 10 mM 2-mercaptoethanol. The dialyzed enzyme was clarified by centrifugation and applied to a 7.5 × 30 cm column of DE-52 cellulose. The column was developed with a gradient of 10–60 mM potassium phosphate, pH 7.0, with a total volume of 4 L. Fractions containing a specific enzyme activity of 100 and above were combined and precipitated with ammonium sulfate at 50% saturation. After centrifugation, the pellet was dissolved in 10 mL of 10 mM Tris, pH 7.0, containing 10 mM 2-mercaptoethanol and dialyzed against the same buffer. The protein solution was applied to a 2.5 × 27 cm hydroxylapatite column (Bio-Gel HT) which was washed with two column volumes of 6 mM potassium phosphate buffer. The enzyme was eluted with a 1-L gradient of 6–100 mM potassium phosphate containing 10 mM 2-mercaptoethanol, pH 7.0. Fractions of specific activity above 600 were combined and concentrated to 15 mL on an Amicon Diaflo apparatus fitted with a PM-30 membrane. Solid ammonium sulfate was added to 50% saturation and the solution centrifuged. The pellet thus obtained was extracted successively with 5 mL each of 40, 35, and 30% saturated ammonium sulfate in 0.1 M potassium phosphate, pH 7.0, containing 1 mM EDTA and 2 mM dithiothreitol. The protein in the extracts crystallized on standing at room temperature with the main crop being in the 35% extract.

Substrates and Radioisotope Determination. All substrates were prepared and radioactivity was determined as described previously (Brems & Rilling, 1979).

Protein Determination. Protein concentration was estimated either by the ninhydrin method (Moore & Stein, 1954) following alkaline hydrolysis or by the spectrophotometric method, using a molar extinction coefficient of 88 400 L cm^{-1} mol^{-1} as determined by Reed (1976).

Preparation of o-Azidophenyl[1-^{3}H]ethyl Pyrophosphate. Because of the unavailability of starting material for synthesizing *o*-azidophenylethanol (Brems & Rilling, 1979), a new and simpler synthesis was devised for this compound. *o*-Nitrophenylethyl alcohol (1 g) was stirred with anhydrous pyridine (150 mL) at 15 °C. Dipyridine–chromium(VI) oxide complex (Collins, 1968) (6 M excess) dissolved in 290 mL of dichloromethane was added in small portions and allowed to react for 90 min. The extent of reaction was monitored by thin-layer chromatography on silica gel plates (Eastman, with fluorescent indicator) with 5% ethyl acetate in toluene as solvent. *o*-Nitrophenylacetaldehyde has an R_f of 0.63; for *o*-nitrophenylethyl alcohol, R_f = 0.08. The reaction was terminated by removal of solvent by rotary evaporation. The products were extracted into diethyl ether, at which time the brownish black, polymeric chromium reduction products were removed by centrifugation. The reaction products were separated on a column of silica gel (Woelm-ICN) with 5% ethyl acetate in toluene as solvent. A yield of 30% was obtained. An excess of *o*-nitrophenylacetaldehyde (0.60 mmol) was reduced by 0.11 mmol of [^{3}H]$NaBH_4$ (25 mCi). *o*-Nitrophenyl[1-^{3}H]ethyl alcohol thus obtained was reduced to *o*-aminophenyl[1-^{3}H]ethyl alcohol by stirring with concentrated hydrochloric acid (1.33 mL) and stannous chloride (0.62 g) at room temperature for 15 h. NaOH (4 N) was added until the white tin complex just dissolved. The solution was extracted with ether, and the extract was washed with water, dried (Na_2SO_4), filtered, and evaporated to give a yellow oil (80 μmol). This was immediately converted into the azide (75 μmol) (Smolinsky & Feuer, 1964; Norman & Radda, 1961). The pyrophosphate ester of *o*-azidophenyl[1-^{3}H]ethanol was prepared as described previously (Brems & Rilling, 1979).

Photoaffinity Labeling. For removal of ammonium sulfate, the protein was chromatographed on a Sephadex G-25 column equilibrated with 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) buffer, pH 7.0, containing 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 100 mM KCl or was dialyzed against the same buffer at 4 °C. 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 the enzyme and analogue were photolyzed at room temperature under a nitrogen atmosphere in a 1-cm quartz cuvette situated between two Mineralights (UVS-11, maximum emission 254 nm). Typically, the enzyme was irradiated with 30 mol of aryl azide/mol of enzyme for four 1-min intervals. After each irradiation, the amount of affinity label was restored to the original concentration and the process repeated. The extent of photoaffinity labeling was monitored by the loss of enzyme activity. Under these conditions, labeling resulted in 80% ± 5% inactivation of enzymatic activity. Irradiation of enzyme under nitrogen in the absence of affinity reagent resulted in

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; Cl_3CCOOH, trichloroacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

no loss of enzymatic activity even after four sequential 1-min irradiations.

Carboxymethylation. Protein was carboxymethylated in 8 M urea by the method of Crestfield et al. (1963). Carboxymethylated protein was then dialyzed exhaustively against distilled water and lyophilized.

Maleylation and Unblocking. Carboxymethylated CNBr peptides were dissolved in 0.1 M $Na_4P_2O_7$ and 6 M guanidine hydrochloride, pH 9.0, to give a concentration of 5 mg mL^{-1}. The peptides were then treated with maleic anhydride (12.5 mg mL^{-1} protein solution) which was added slowly as a solid. The pH was maintained at 8.5–9.0 by the addition of 1.0 N NaOH until the pH stabilized. The solution was then desalted by chromatography on Sephadex G-25. The number of maleyl groups bound to the peptides was estimated spectrophotometrically as described by Butler & Hartley (1972). Maleyl groups were removed by incubating the peptides in a solution of 8% acetic acid and 2% formic acid in water, pH 2.1, at 50 °C for at least 24 h.

Tryptic Peptide Maps. A modification of the method of Katz et al. (1959) was used. Carboxymethylated protein (5 mg mL^{-1}) was treated with trypsin (1.5% of the protein by weight). After lyophilization, 200 μg of the digest was applied to an Eastman "Chromogram" cellulose thin-layer sheet. Ascending chromatography was with 60% 1-butanol, 15% glacial acetic acid, and 25% water as solvent. Electrophoresis at pH 3.5 in 0.3% pyridine, 3.3% glacial acetic acid, and 96.4% water was then perpendicular to the direction of chromatography and was at 1400 V, 11–18 A for 60 min. Peptides were located by fluorescamine (Felix & Jiminez, 1974) or cadmium–ninhydrin (Whitaker, 1967).

Fluorography. For fluorography, the tryptic map was dipped in a 25% solution of 2,5-diphenyloxazole in acetone. After the thin-layer sheet dried, it was exposed to Kodak X-Omat R film for 2–10 days at −60 °C.

Cyanogen Bromide Cleavage. A 100-fold excess of CNBr was added to carboxymethylated protein (5–10 mg mL^{-1}, in 70% formic acid. After 24 h, the reaction was terminated by dilution with water and followed by lyophilization.

Analytical Polyacrylamide Gel Electrophoresis. Analytical gels were by the method of Swank & Munkres (1971). The distribution of ^3H in the gel was determined by dissolving 2-mm segments of the gel in perchloric acid–H_2O_2 (Mahin & Lofberg, 1966). The solubilized gel segments were analyzed for radioactivity by liquid scintillation spectrometry.

Peptide Purification. The maleylated CNBr peptides from photolabeled protein were chromatographed on a 1.5 × 100 cm column of Sephadex G-100 with 0.5 M NH_4HCO_3, pH 9.0, as solvent. Fractions were monitored for the absorbance at 280 nm and/or radioactivity. Combined radioactive fractions were lyophilized and then applied to a 0.5 × 15 cm column of DEAE-cellulose equilibrated with 0.05 M Tris-HCl, pH 8.6. The column was developed with a linear gradient of 0.05 M Tris-HCl, pH 8.6, to 0.05 M Tris-HCl, pH 8.6, containing 0.8 M NaCl (400-mL total volume). Pooled radioactive fractions were desalted by chromatography on a 1.5 × 60 cm column of Sephadex G-25 with 0.6 M NH_4HCO_3, pH 9.0, as solvent. The purified peptide was then unblocked. If the peptide was destined for sequence analysis, it was incubated with alkaline phosphatase (Sigma, calf intestine type VII) at a 1:1 ratio (w/w) for 1 h in 0.1 M NH_4HCO_3 with 1 mM $MgCl_2$, pH 9.0. Removal of the pyrophosphate moiety from the modified peptide allowed for more efficient extraction of the modified thiazolinone liberated after each Edman degradation step.

Due to the different properties of the CNBr peptides from those of the native protein, they could not be isolated by the procedure described for the labeled CNBr peptide. The following protocol was utilized for isolation of native CNBr fragments. CNBr peptides were separated into excluded and retarded fractions by passage over a 1 × 60 cm column of Sephadex G-25 in 60% formic acid. Excluded peptides which represent more than 80% of the applied sample were then resolved by preparative polyacrylamide gel electrophoresis by the method of Swank & Munkres (1971).

Following electrophoresis, gels were stained by soaking for at least 2 h in a solution containing 0.2% Coomassie Brilliant Blue G (Sigma) in 45% methanol, and 10% acetic acid in water. Destaining was effected by diffusion in a solution of 10% methanol and 7% acetic acid in water. Appropriate bands were excised and homogenized in a Potter Elvehjem homogenizer with 60% formic acid as described by Gibson & Gracy (1979). Polyacrylamide fines were removed by filtration through Sephadex G-25 in 60% formic acid. After lyophilization, Coomassie Brilliant Blue and $NaDodSO_4$ were extracted into a mixture of acetone–triethylamine–acetic acid–water, 85:5:5:5, as described by Henderson et al. (1979). After removal of dye and $NaDodSO_4$, the peptide was washed twice with acetone and dried.

End-Group Determination. The amino-terminal amino acid was determined by dansylation (Gray, 1967). Dansyl amino acids were analyzed by high-voltage electrophoresis at pH 4.4 or 1.9 as described by Gray (1967).

Amino Acid Analysis. Purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 100 °C for 24 h in vacuo. Amino acid compositions were determined on a Beckman 120 C analyzer with a single-column procedure (Spackman et al., 1958).

Pronase Digestion. Peptide 8-P was incubated with Pronase (Sigma) in 0.1 M NH_4HCO_3, pH 7.8, for 24 h. The digest was lyophilized and analyzed by two-dimensional thin-layer chromatography on a 20 × 20 cm thin-layer silica gel plate (Merck) with chloroform–methanol–ammonia–water, 40:40:4:16, in the first dimension followed by phenol–water, 75:25, in the second.

Edman Degradation. Automated Edman degradation was with a Beckman Model 890 C with 0.1 M Quadrol as the coupling buffer and Beckman program 121078. The spinning cup was pretreated with polybrene (Aldrich Chemical Co.) according to the method of Hunkapiller & Hood (1978), and conversion of thiazolinone derivatives was carried out in 0.2 N methanolic HCl with a Sequemat P-6 autoconverter. Phenylthiohydantoin (Pth) derivatives were identified by high-pressure liquid chromatography according to Margolies & Brauer (1978) with a μ-C_{18} reverse-phase column (Waters) at 55 °C with a linear gradient of 27–50% acetonitrile in 20 mM sodium acetate buffer, pH 5.0. Pth derivatives also were identified by amino acid analysis after hydrolysis by 6 N HCl (24 h at 150 °C) (Van Orden & Carpenter, 1964).

Results

Tryptic Maps. To determine the number of peptides that had become photolabeled, we digested the derivatized enzyme with trypsin. A total of 43 peptides were detected consistently. When resulting tryptic maps were analyzed by fluorography, two and perhaps three labeled peptides were detected. Two peptides chromatographed with R_f values of 0.64 and 0.82 but did not migrate during electrophoresis. The third radioactive zone remained at the origin and may be core protein. None of the fluorescamine-positive spots were radioactive. The

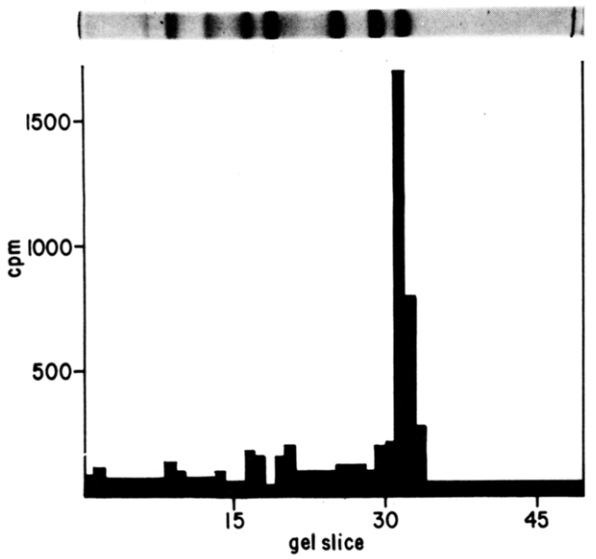

FIGURE 1: Photoaffinity-labeled enzyme was cleaved with CNBr and subjected to $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. Gels were sliced into 2-mm segments, and radioactivity was determined. $NaDodSO_4$–urea–polyacrylamide gel electrophoresis profiles of native CNBr peptides have identical staining patterns.

radioactivity recovered from the origin (35%) and the other two radioactive spots (55%) consisted of nearly all of the radioactivity originally applied.

In an attempt to distinguish between the allylic and homoallylic sites, the enzyme was photoaffinity labeled in the presence of saturating amounts of the allylic substrate geranyl pyrophosphate. Fluorography of the resulting tryptic map showed a marked decrease in the radioactive spot that migrated furthest from the origin.

Cyanogen Bromide Peptides. Since prenyltransferase contains seven methionine residues, cleavage with CNBr should yield eight peptides. Indeed, analysis by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of CNBr-treated enzyme indicates eight clearly distinguishable bands. These peptides are numbered according to their relative position on the gel, starting with the peptide nearest the origin (Figure 1). CNBr peptides obtained from labeled and native enzyme are identical as judged by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. Gels were sliced, and the radioactivity of each slice was determined. The results shown in Figure 1 demonstrate that only peptide 8 is significantly photoderivatized.

Purification of Labeled Peptide. A 9-mg sample of enzyme, labeled to the extent of 80% loss of enzymatic activity, was reduced, carboxymethylated, cleaved by CNBr, and maleylated. The peptides thus obtained were chromatographed on Sephadex G-100, and the elution profile obtained is shown in Figure 2. Three peaks were observed. The third peak, which represented 70% of the radioactivity applied, contained peptides 6, 7, and 8 as judged by polyacrylamide gel electrophoresis. These peptides were then separated by chromatography on DEAE-cellulose. Figure 3 shows the elution profile obtained. The first peak is peptide 7, the second peak is peptide 8, and the third peak is peptide 6, as determined by migrations identical with those of native peptides 6, 7, and 8 on $NaDodSO_4$–urea–polyacrylamide gels. The second peptide peak, which contained over 90% of the radioactivity eluted from DEAE and 65% of that applied, corresponded to native peptide 8 and will be referred to as peptide 8-P. The purity of each peptide was 95% as judged by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis, NH_2-terminal analysis, and amino acid analysis.

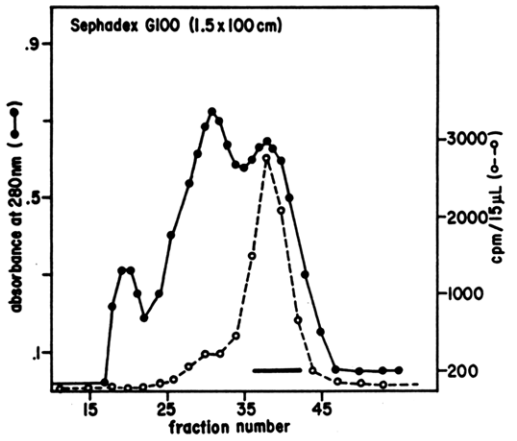

FIGURE 2: Separation of photoaffinity-labeled CNBr peptides on a Sephadex G-100 column. Fractions were collected, and the absorbance at 280 nm was measured (solid line). Aliquots of 15 μL were used for radioactivity determination (dashed line). Fractions which were pooled and eventually sequenced are indicated by a solid bar.

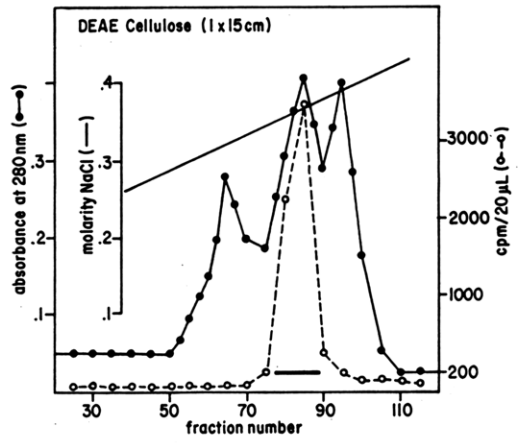

FIGURE 3: Anion-exchange chromatography on DEAE-cellulose of photoaffinity-labeled CNBr peptide fraction from Sephadex chromatography. The column was developed with a linear gradient. Fractions were collected, and the absorbance at 280 nm was measured (solid line). Aliquots of 20 μL were used for radioactivity determination (dashed line). Fractions which were pooled and eventually used for sequence analysis are indicated by a solid bar.

Purification of Native Peptides 6, 7, and 8. CNBr peptides that had previously been reduced and carboxymethylated were purified by preparative electrophoresis in polyacrylamide gels. Peptides were eluted from the gel with a recovery of 45%. The purity of these peptides (6, 7, and 8) was established by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis, NH_2-terminal analysis, and compositional analysis and was better than 80%.

End-Group Analysis. Dansylation of peptide 8-P, isolated by chromatography, revealed only one α-amino acid derivative; *N*-dansylleucine, *o*-dansyltyrosine, dansylamide, dansyl hydroxide, and N^{ϵ}-dansyllysine were all present and identified. End-group determination of native peptide 8 and labeled peptide 8-P isolated by preparative $NaDodSO_4$–urea–polyacrylamide gel electrophoresis yielded the same information.

Amino Acid Composition. Amino acid analyses of peptides 8-P and 8 were determined and are given in Table I. Cysteine was measured as its carboxymethyl derivative. All hydrolysates contained small amounts of homoserine and homoserine lactone, but these amino acids were not quantitated. Table I shows that the amino acid compositions of CNBr peptide 8-P purified as the maleylated peptide are identical with those of CNBr peptide 8, which was purified from native protein

Table I: Comparison of Amino Acid Composition of Labeled and Native Peptide[a]

amino acid	8-P analysis	analysis of native peptide 8	8-P sequence
Asp	2.2	2.0	2
Thr	1.2	1.0	1
Ser	1.7	2.1	2
Glu	3.2	3.5	3
Pro	1.7	1.4	2
Gly	1.3	0.7	1
Ala	2.8	3.2	2
Val	2.6	2.0	3
Ile	1.0	1.0	1
Leu	2.9	2.6	3
Tyr	1.9	2.2	2
Phe	1.6	2.4	2
His			
Lys	2.8	2.5	3
Arg	1.0	1.0	1
Cys			

[a] The amino acid compositions were obtained from a 24-h 6 N HCl hydrolysis of the peptides. Peptide 8-P represents CNBr peptide isolated from photoaffinity-labeled prenyltransferase by molecular sieving and ion-exchange chromatography as described in the text. Native peptide 8 represents CNBr peptide isolated from native prenyltransferase by preparative gel electrophoresis. Cys was determined as its carboxymethyl derivative.

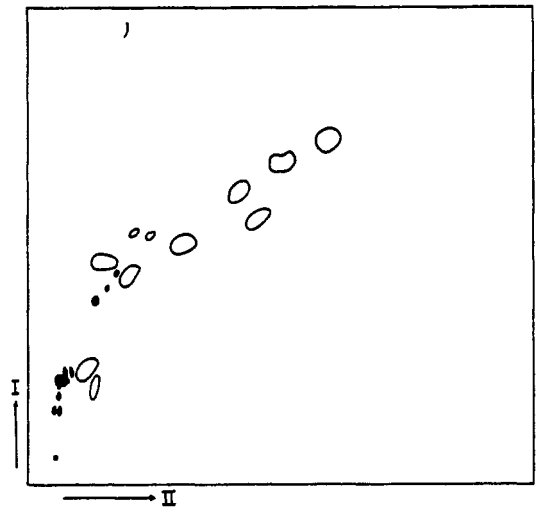

FIGURE 4: Two-dimensional thin-layer chromatography of a Pronase digest of photoaffinity-labeled CNBr 8-P. A 10-nmol sample of photolabeled peptide 8-P was digested with Pronase and developed by two-dimensional thin-layer chromatography. Detection was with ninhydrin followed by fluorography. The black spots indicate radioactivity.

by polyacrylamide gel electrophoresis.

Pronase Digestion of Peptide 8-P. For determination of the number of residues that had been modified by photoaffinity labeling, peptide 8-P was digested extensively with Pronase to yield free amino acids. The resulting digest, when analyzed by two-dimensional thin-layer chromatography (Figure 4), revealed at least 11 radioactive spots. None of the radioactive zones corresponded to ninhydrin-positive material. A Pronase digest of native CNBr peptide 8 gave a pattern of ninhydrin-positive spots identical with that of peptide 8-P. Each ninhydrin-positive area could be identified as an amino acid by comparison with standards.

Trypsin Digestion of Peptide 8-P. When peptide 8-P was digested with trypsin, the distribution of radioactivity was identical with that found for the tryptic digest of labeled prenyltransferase. As before, none of the radioactivity was associated with the ninhydrin-positive regions.

Table II: Sequence of Native CNBr Peptide

step no.	Pth-amino acid	8[a] % yield	8-P[b] % yield
1	Leu	26	31
2	Asp	20	62
3	Leu	76	96
4	Ile	88	80
5	Gly	19	25
6	Ala	98	60
7	Pro	66	40
8	Val	75	61
9	Ser[c]		
10	Lys	37	30
11	Val	52	42
12	Asp	33	28
13	Leu	37	27
14	Ser[c]		
15	Thr	9	18
16	Phe	63	25
17	Gln	11	10
18	Glu	18	14
19	Glu	26	20
20	Arg	15	20
21	Tyr	20	10
22	Lys	11	14
23	Ala	14	9
24	Phe	16	10
25	Val	11	13
26	Pro	20	6
27	Tyr	12	12
28	Lys	12	8
29	Ala	10	5
30	Met[d]	9	8

[a] Native CNBr peptide 8 (100 nmol) was analyzed in the Beckman Model 890C. Identification of the Pth-amino acids liberated at each step of Edman degradation was made by high-pressure liquid chromatography (HPLC) and amino acid analysis of back-hydrolysates. See text for details. [b] Photoaffinity-labeled CNBr peptide 8-P (80 nmol) was analyzed in the Beckman Model 890C. Identification of the Pth-amino acids liberated at each step of Edman degradation was made by HPLC and amino acid analysis of back-hydrolysates. See the text for details. [c] The Pth-amino acid liberated at this step could not be identified. It was assumed to be Ser from the amino acid composition, since all other residues were accounted for. [d] Methionine was determined as the sum of homoserine plus homoserine lactone.

Sequence Determination. Sequence analysis by automated Edman degradation of native CNBr peptide 8 yielded all 30 residues. The results obtained are summarized in Table I which shows the sequence is Leu-Asp-Leu-Ile-Gly-Ala-Pro-Val-Ser-Lys-Val-Asp-Leu-Ser-Thr-Phe-Gln-Glu-Glu-Arg-Tyr-Lys-Ala-Phe-Val-Pro-Tyr-Lys-Ala-Met.

Sequence analysis of peptide 8-P also gave 30 residues. The results (Table II) show the sequence to be Leu-Asp-Leu-Ile-Gly-Ala-Pro-Val-Ser-Lys-Val-Asp-Leu-Ser-Thr-Phe-Gln-Glu-Glu-Arg-Tyr-Lys-Ala-Phe-Val-Pro-Tyr-Lys-Ala-Met. An aliquot (20%) of the Pth-amino acid liberated at each step of the Edman degradation was analyzed for radioactivity. The results are illustrated in Figure 5. A total of 16 individual steps released significant amounts of radioactivity. After 30 steps, 60% of the total radioactivity was recovered.

Discussion

Initially, isolation of labeled tryptic peptides from photoderivatized enzyme was attempted after trypsin digestion. Although isolation and characterization of these peptides were not successful, considerable information concerning the nature and extent of photoaffinity labeling was obtained. The tryptic maps of enzyme affinity labeled to the extent of 0.6 mol of analogue per catalytic site had at least three radioactive spots. One of these remained at the origin. The other two migrated

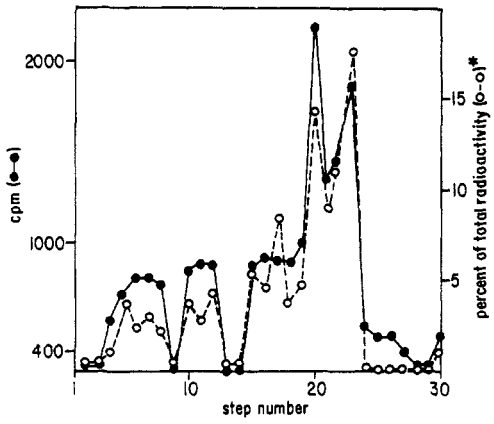

FIGURE 5: Recovery of radioactivity from Edman degradation of labeled CNBr peptide 8-P. An 80-nmol sample (1.6×10^6 dpm) of photolabeled CNBr peptide 8-P was subjected to automated sequence analysis. One-fifth of the Pth-amino acids liberated at each step of the Edman degradation were analyzed for radioactivity (solid line). The dashed line represents the percent total radioactivity after correction for a repetitive yield of 93% and an initial yield of 30% in addition to a correction for carry-over from previous steps.

in the first dimension (chromatography) but remained at the electrophoretic origin. The two radioactive spots which migrated did not coincide with any fluorescamine-positive areas and were diffuse. These results are interpreted to indicate that the affinity label had reacted with more than one amino acid. The absence of fluorescamine-positive staining associated with the radioactive spots may result from the diffuse nature of these regions. When geranyl pyrophosphate was included during the labeling process, the radioactive spot which migrated furthest away from the origin was greatly diminished. Thus, apparently this peptide is principally associated with the allylic site.

All other attempts to isolate radioactive tryptic peptides failed. Ion-exchange chromatography resulted in broad radioactive elution profiles, confirming the heterogeneity suggested by the tryptic maps.

Since chromatography of the tryptic peptides on DEAE-cellulose did not reveal discrete bands of radioactivity, we resorted to procedures that would yield larger peptides. Analysis of the number of peptides with attached affinity reagent revealed that only one of the eight CNBr peptides (8-P) was significantly labeled (Figure 1). Over 80% of the recovered radioactivity was located in peptide 8-P. Isolation of this radioactive peptide was not easily achieved, chiefly due to the insolubility and aggregating nature of the CNBr peptides. Key to the isolation of peptide 8-P was maleylation, which rendered the peptides soluble in aqueous buffers and consequently more amenable to purification by conventional chromatographic techniques. However, with this purification procedure, it was not possible to isolate the corresponding native CNBr peptide. For this reason, native CNBr peptide 8 was isolated by preparative polyacrylamide gel electrophoresis.

End-group analysis of peptide 8-P disclosed leucine as the amino-terminal amino acid and allowed an estimation of purity of at least 90%. No significant differences were observed in amino acid analysis of labeled and native CNBr fragments isolated by the two different techniques, confirming that they are identical peptides. There were no unusual peaks obtained on amino acid analysis of peptide 8-P. Therefore, either hydrolysis in 6 N HCl at 110 °C cleaves the bond linking label to the peptide, thus regenerating the original amino acid, or the label attached to the peptide is distributed among several amino acids, rendering them undetectable.

Two-dimensional chromatography of Pronase digests of peptide 8-P ascertained a number of amino acids had been modified by photoaffinity labeling. Pronase will cleave a peptide to its free amino acids under conditions which do not modify the attached affinity label. Fluorography of the resulting chromatograms indicated the presence of 11 radioactive regions (Figure 4). Thus, at least 11 different amino acids or peptides were labeled. Since none of the radioactive spots are ninhydrin positive, only a small fraction of any one amino acid is modified by the labeling procedure. All ninhydrin-positive zones were identified as unmodified amino acids.

Tryptic maps of peptide 8-P showed one radioactive spot at the origin and two other diffuse spots with R_f values identical with those observed in tryptic maps of the photolabeled enzyme. The identical pattern of distribution of radioactivity in the trypsin maps of peptide 8-P and the photoaffinity-labeled enzyme verifies that the majority of label is carried in peptide 8-P.

The peptide of interest was also analyzed by Edman degradation. Prior to degradation, it was necessary to remove the pyrophosphate residue, since, if the alkaline phosphatase treatment was omitted, less than 5% of the radioactivity applied could be accounted for. After preincubation with alkaline phosphatase, over 90% of the expected radioactivity was recovered. Apparently, the low pK and polarity of the pyrophosphate moiety results in poor partitioning in the butyl chloride extraction.

For the purpose of discussion, the radioactivity released can be divided into five regions. The first region consists of the five amino acids Ile-Gly-Ala-Pro-Val which contains approximately 10% of the total radioactivity. The prominent feature of this segment is its hydrophobic nature. This region could serve as a hydrophobic pocket or portion of a pocket for the hydrocarbon moieties of the substrates. The second region also contains 10% of the total radioactivity and consists of three amino acids, Lys-Val-Asp. The third region is the four amino acids Thr-Phe-Gln-Glu, representing 20% of the total radioactivity. The fourth region is made up of the three amino acids Glu-Arg-Tyr and contains 30% of the radioactivity. These amino acids are polar with two being ionic. Associated with the step that releases arginine is 15% of the radioactivity, which is interesting since Barnard & Popjak (1980) reported the presence of an essential arginine in the catalytic site of pig liver prenyltransferase. Many enzymes whose substrates are phosphate esters contain arginine residues that are essential for catalysis or binding of substrates (Riordan et al., 1977). The presence of arginine in the catalytic site of prenyltransferase further substantiates this arginine–pyrophosphate postulate. With [^{14}C]phenylglyoxal, at least ten arginines of the avian liver enzyme reacted with this reagent (Brems, 1980), whereas, under identical conditions, Barnard and Popjak found only two residues modified. Differential labeling, by first protecting the active-site arginine with saturating levels of substrate during reaction with phenylglyoxal followed by reaction with [^{14}C]phenylglyoxal, failed to specifically label the catalytic-site arginine (Brems, 1980). Differential labeling yielded one-half the total number of [^{14}C]phenylglyoxals bound (Table III). Since two phenylglyoxals will react with one guanido group (Takahashi, 1968), it appears that differential labeling simply equilibrated all the exchangeable phenylglyoxals with the radioactive reagent.

The last region of radioactivity (30% of the total) consists of the three amino acids Lys-Ala-Phe. Thus, a total of 16 of 30 amino acids of peptide 8-P contain significant radioactivity. Seven of these are nonpolar, accounting for 35% of the total

Table III: Stoichiometry of Binding of [^{14}C]Phenylglyoxal to Prenyltransferase[a]

% inactivation	9	20	31	45	53	61	80
mol of [^{14}C]phenylglyoxal bound/mol of enzyme monomer	2.6	5.1	7.9	11.1	13.0	15.0	20.0

[a] Prenyltransferase was inactivated by varying the times of incubation with [^{14}C]phenylglyoxal to obtain the fractional inactivation as shown in the table. At varying times 200-μL aliquots were removed and used for determination of binding of the ^{14}C-labeled reagent to the protein, and 10-μL aliquots were assayed for residual enzyme activity.

radioactivity. Three of the 16 are polar but uncharged, containing 25% of the total. Six of the 16 are ionic and represent 40% of the total radioactivity. The carboxyl end of region 3, all of region 4, and the amine end of region 5, Gln-Glu-Glu-Arg-Tyr-Lys, consist of all changed or polar amino acids.

Photoaffinity reagents are useful for analysis of complex molecules since they are converted to reactive forms only on exposure to activating light. The nitrene(s) generated from the substrate analogue used in these experiments can react with solvent or with the individual amino acids of the protein. Since peptide 8-P was isolated by chromatographic procedures which included exposure to 60% formic acid, the linkage of this reagent to the different amino acid(s) is moderately stable (Brems, 1980). This observation is consistent with results obtained in other systems (Bayley & Knowles, 1977). Digestion of this peptide with Pronase yielded 11 discrete radioactive compounds, indicating that as many as 11 different residues had been attacked by the reagent. However, since it is possible that several different products could be obtained by the reaction of the activated reagent with any one amino acid, 11 may be an upper limit for the number of amino acids effected.

Degradation by the Edman procedure which utilizes heptafluorobutyric acid for cleavage also demonstrated a nonselective distribution of the affinity reagent. In addition, acid hydrolysis revealed no selective modification of one or a few amino acids. However, one must be cautious since it is possible the derivative(s) formed could regenerate the original amino acid on acid hydrolysis. However, the evidence, taken together, strongly suggests a diffuse rather than a selective distribution of modified amino acid(s).

The results from the Edman degradation are indicative of the distribution of the label in the region of the catalytic site (peptide 8). One can envision an active site constructed in two ways. In one, the catalytic pocket would consist of amino acids, perhaps distant in their primary structure but juxtaposed by tertiary structure. In another, the active site would be composed of amino acids closely related in their primary structure with the appropriate secondary and tertiary structures to accommodate binding of substrates. Indeed, active-site structures deduced by X-ray diffraction studies have shown that both possibilities exist with a predominance of the former. Furthermore, many catalytic sites of multisubunit enzymes have been located within the cleavage of two subunits. None of the data presented herein support a catalytic site composed of amino acids of distant primary structure juxtaposed by tertiary structure, nor a catalytic site located within the cleavage of two subunits. However, it is not possible to conclude that the catalytic site is entirely composed of a single peptide (16 residues of CNBr peptide 8-P) because of the limitations of the technique. Namely, the predictable half-life of the nitrene generated at the catalytic site compared with the rate of diffusion provides ample time for movement within the catalytic site which may allow the activated nitrene to be selective with respect to which amino acid it attacks. In addition, there may be certain binding constraints imposed upon the photoaffinity label which would exclude its reaction with particular amino acids or preclude its reaction with partial segments of the catalytic site. Thus, there may indeed be some amino acids present within the catalytic site that may be far distant in primary structure to peptide 8-P.

Since experimental evidence has shown that the conformation of proteins is determined predominantly by short-range interactions (i.e., single residue information and neighboring residue information), the sequence of CNBr fragment 8 was subjected to protein conformational analysis. The popular model of Chou & Fasman (1974a) was used for analysis of this peptide. Only the decapeptide Val-Asp-Leu-Ser-Thr-Phe-Gln-Glu-Glu-Arg satisfies the requirements for predicted helices since it has a helical potential, $\langle P_\alpha \rangle$, of 1.12, which is above the minimum potential for expected helices. The helical nature of this decapeptide is of special interest since this ten amino acid fragment contains 50% of all the photoaffinity label associated with the peptide. The findings of Nakanishi et al. (1972) that the central core of the α helix has greater stability than the terminal portions led them to conclude that considerable flexibility exists at the helix–coil border region which diminishes sharply as the inner helical core is approached. This led Chou & Fasman (1974b) to speculate that the propensity for polar residues at helix–coil boundaries makes these regions more flexible than the rigid inner helix core, thus facilitating substrate binding as well as catalysis. It also is possible that the polar residues found at conformational junctions serve as signals for protein folding. The three C-terminal amino acids of this peptide are polar and charged and contain approximately 50% of the total label associated with the helical decapeptide. Thus, it appears that Glu-Glu-Arg, located on the flexible C terminus of the helix, may play a role in binding to substrate pyrophosphate and perhaps in catalysis.

An α-helix model was built to represent the predicted helical decapeptide (residues 11–20) by use of the CPK space-filling atomic models. The model indicates the presence of a hydrophobic cleft composed of the hydrocarbon portions of the side chains of arginine-20 and glutamine-17, along with the side chains of Phe-16 and Val-11. CPK space-filling models of either the allylic or the homoallylic substrates fit in the groove with their pyrophosphate moieties contacting the guanidinium group of Arg-20 from one side and the amide nitrogen of glutamine-17 from the opposite side.

The only portion of peptide 8 that might be a β sheet is the N-terminal pentapeptide Leu-Asp-Leu-Ile-Gly. It has a β-sheet parameter potential, $\langle P_\beta \rangle$, of 1.13, which again is above the minimum potential expected for β-sheet structure. This β-sheet pentapeptide accounts for 3% of the total label attached to CNBr peptide 8-P.

There is a potential β turn at amino acids 25–28 (Val-Pro-Tyr-Lys) with a β-turn conformational parameter, P_f, of 0.503×10^{-4}, which is above the cutoff value for predicting β turns. The radioactivity associated with CNBr 8-P terminates with amino acid residue 25, the first amino acid of the β turn. The lack of attached label at Val-25 and throughout the remainder of the peptide is explained by the 180° chain reversal caused by the tetrapeptide β turn. The β bend would result in hydrogen bonding between the carbonyl group of residue i (Val) and the NH_2 group of residue $i + 3$ (Tyr) (Venkatacholam, 1968), thus removing this portion of the peptide chain from contact with the activated nitrene generated at the catalytic site.

In summary, the active site of farnesyl pyrophosphate synthetase has been selectively modified with a catalytic site directed photoaffinity label. A CNBr fragment of 30 amino acid residues from the photoaffinity-labeled enzyme has been isolated and characterized. This CNBr fragment contains over 80% of the total label attached to prenyltransferase as a result of photoaffinity labeling. Several lines of evidence indicate that a number of amino acids from this CNBr fragment have been modified. First, peptide maps of a trypsin digest of this CNBr fragment indicate that the attached affinity label is distributed among at least three of the resulting tryptic peptides. Second, comparison of amino acid analysis of this CNBr fragment with its counterpart isolated from native enzyme also suggests that a number of amino acids have been modified rather than a few by the photoaffinity labeling process. Third, two-dimensional chromatography of a Pronase digest of the labeled CNBr fragment indicates that at least 11 different amino acid products resulted from photoaffinity labeling. Finally, Edman degradation of this labeled peptide demonstrates that at least 16 of the 30 amino acids have been modified by the photoaffinity reagent. The two most frequently modified amino acids are a specific arginine and alanine.

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