Prenylated proteins contain either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid covalently attached to cysteine residues at or near their C terminus. The cellular abundance of prenylated proteins, as well as the stability of the thioether bond, poses a metabolic challenge to cells. A lysosomal enzyme termed prenylcysteine lyase has been identified that degrades a variety of prenyl cysteines. Prenylcysteine lyase is a FAD-dependent thioether oxidase that produces free cysteine, an aldehyde, and hydrogen peroxide as products of the reaction. Here we report initial studies of the kinetic mechanism and stereospecificity of this unusual enzyme. We utilized product and dead end inhibitors of prenylcysteine lyase to probe the kinetic mechanism of the multistep reaction. The results with these inhibitors, together with those of other experiments, suggest that the reaction catalyzed by prenylcysteine lyase proceeds through a sequential mechanism. The reaction catalyzed by the enzyme is stereospecific, in that the pro-S hydride of the farnesylcysteine is transferred to FAD to initiate the reaction. With \( \frac{2}{H} \text{farnesylcysteine} \) as a substrate, a primary deuterium isotope effect of 2 was observed on the steady state rate. However, the absence of an isotope effect on an observed pre-steady-state burst of hydrogen peroxide formation implicates a partially rate-determining proton transfer after a relatively fast C–H (C–D) bond cleavage step. Furthermore, no pre-steady-state burst of cysteine was observed. The finding that the rate of cysteine formation was within 2-fold of the steady-state \( k_{\text{cat}} \) value indicates that cysteine production is one of the primary rate-limiting steps in the reaction. These results provide substantial new information on the catalytic mechanism of prenylcysteine lyase.

Protein prenylation is a type of posttranslational lipid modification involving the covalent attachment of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid via thioether linkages to a conserved cysteine of many cellular proteins. The majority of prenylated proteins participate in biological regulatory events occurring at the cytoplasmic surface of cell membranes. Most prenylated proteins contain the so-called CaaX motif, in which the “C” is the modified cysteine, the “a” residues are usually aliphatic amino acids, and the X residue can be one of several amino acids. Following prenylation, most CaaX-type proteins are subject to two additional processing steps: the three C-terminal residues are proteolytically removed, and the new C-terminal prenylcysteine is subject to carbonyl methylation (3–5).

Unlike many other posttranslational modifications of proteins, prenylation is a stable modification of proteins (6, 7). Prenylated proteins comprise up to 2% of total cellular protein and have an approximate half-life of 20 h (6, 7); however, accumulation of prenylcysteines arising from degradation of prenyl proteins is expected to place a major burden on cells. Since free prenylcysteines and analogs have a number of pharmacological effects on cells (8–11), the mechanisms for cellular disposal of the prenylcysteines produced during the normal turnover of prenylated proteins are important to establish.

Studies aimed at elucidating the metabolism of prenylated proteins resulted in the identification of a lysosomal enzyme that catalyzes the degradation of prenylcysteines and their methyl esters (12, 13). This enzyme has been termed prenylcysteine lyase (Pcly).1 The basic features of the chemical mechanism through which Pcly catalyzes its reaction have been elucidated (14). The main features are as follows: (i) the products of the reaction are free cysteine and the C-1 aldehyde of the isoprenoid moiety (farnesal or geranylgeranal); (ii) the enzyme utilizes molecular oxygen as a co-substrate; (iii) Pcly uses a noncovalently bound FAD cofactor in an NAD(P)H-independent manner; and (iv) a stoichiometric amount of hydrogen peroxide is produced during the course of the reaction. The last point presumably indicates that no oxygen atom from the molecular oxygen is incorporated into the farnesal product. A chemical mechanism for Pcly that can account for the above observations has been proposed (14). In the proposed mechanism (Fig. 1), the reaction is initiated by a hydride transfer from C-1 of the isoprenoid moiety of FC to FAD, producing reduced flavin and a probably short-lived sulfur-stabilized carbocation intermediate. Nucleophilic attack of a water molecule on the carbocation results in the formation of a hemithioacetal intermediate, which collapses to the isoprenoid aldehyde with simultaneous C–S bond breakage to produce cysteine. In addition, the hydride of the reduced flavin is transferred to molecular oxygen, resulting in hydrogen peroxide formation and reoxidation of the flavin, thus allowing the enzyme to participate in subsequent turnovers.

1 The abbreviations used are: Pcly, prenylcysteine lyase; FC, farnesylcysteine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GC, gas chromatography.
The mechanism proposed for Pcly (i.e. oxidative cleavage of a thioether bond without net oxidation at sulfur) appears to be unprecedented in biology. The finding that cysteine is produced from cysteines, namely carbon-sulfur-δ-lyase, which would produce a prenylthiol (15). Furthermore, the lack of a NADPH requirement suggests that Pcly uses a mechanism distinct from cytochrome P450 and flavin-containing monooxygenases that require a prenylthiol (15). Furthermore, the lack of a NADPH requirement suggests that Pcly uses a mechanism distinct from cytochrome P450 and flavin-containing monooxygenases that catalyze S-oxidation of FC and other thioethers (16–18). In addition, Pcly has essentially no sequence similarity to known lipidated proteins.

The stereochemistry of the Pcly reaction, three separate incubations were set up in which Pcly used either unlabelled FC, (2R,1’S)-[1-2H1]-FC, or (2R,1’R)-[1-2H1]-FC as a substrate. Reaction mixtures were prepared in glass vials that were prevaswed in hexane and contained the appropriate farneslycysteine (500 μM), 12.5 μM (for the reactions with unlabeled FC and the R-isomer) or 20 μM (for the reaction with the S-isomer) Pcly, 6 mM NADPH (2.5% efficiency), 50 mM sodium phosphate, pH 7.4, 1 unit/ml horseradish peroxidase, and 200 mM Amplex Red reagent at 25 °C. The anomalously high deuterium content in the 2H1-2H1-[1-2H1]-FC, or (2-2H1)-[1-2H1]-FC as a substrate. Fraction containing Pcly activity were pooled and dialyzed against 20 mM Tris-Cl, pH 7.7, and 150 mM NaCl, 8 mM imidazole, and 0.2% CHAPS. Pcly was eluted with a 44-ml linear gradient of imidazole (8–500 mM) in the above buffer. Fractions containing Pcly activity were pooled and dialyzed against 20 mM Tris-Cl, pH 7.7, containing 0.2% CHAPS (buffer A). The dialysate was loaded onto a HiPrep 16/10 column (Amersham Biosciences), which was equilibrated in buffer A. Pcly was eluted with a 120-ml gradient of NaCl from 0 to 500 mM in buffer A. Fractions containing Pcly were pooled, dialyzed as above, and stored at −80 °C.

**Pcly Stereochemistry—**To determine the stereochemistry of the Pcly reaction, three separate incubations were set up in which Pcly used either unlabelled FC, (2R,1’S)-[1-2H1]-FC, or (2R,1’R)-[1-2H1]-FC as a substrate.
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RESULTS

Chemical Synthesis of (2R,1R)- and (2R,1′S)-[1-1H2]Farnesylcysteine—Farnesylcysteines are stereoselectively labeled with deuterium in the pro-R and pro-S positions at C-1 were prepared by asymmetric reductions of farnesal-Δ2H15 with S-Alpine-Borane (Fig. 2) (28, 29). The enantiomeric purities for FC in the corresponding 1H NMR analyses of the corresponding camphane esters (23, 30). Chromatographic purification of the labeled farnesols proved difficult due to persistent contamination by an impurity presumed to be pinanol arising from oxidation of the pinyholane reagent. An effective purification of the S-enantiomer was ultimately accomplished by selective acetylation of farnesol, chromatography, and acetic cleavage. The labeled FC diastereomers were obtained by conversion to the farnesyl chlorides with N-chlorosuccinimide-dimethyl sulfoxide reagent (31) followed by S-alkylation of cysteine (32). Since both reactions are expected to proceed by Sn2 displacements with inversion of configuration (28, 33), the overall stereochemical outcome is retention affording (2R,1′R)– and (2R,1′S)-[1-1H2]FCs. The diastereopurities of 91 and 95%, respectively, determined from their 1H NMR spectra in ethanol-d6, reveal that some racemization occurred, probably in forming and/or handling the labile chloride intermediate.

Stereochemistry of the Pcly Reaction—In the mechanism proposed previously for Pcly (14) (See Fig. 1), a hydride from the C-1 of the isoprenoid moiety of FC is transferred to FAD, forming a sulfur-stabilized carbocation intermediate and reduced flavin. It is expected that either the pro-S or pro-R hydride will be transferred to FAD, whereas the other will be retained in the product. Pcly was incubated with unlabeled FC or with each of the deuterated isomers (see “Experimental Procedures”). After incubation, the reactions were treated with NaBH4 to reduce the farnesal product to the corresponding alcohol, farnesol. The deuterium content in the trans, trans-farnesol was determined by GC-mass spectrometry analysis. The results, summarized in Table I, reveal that Pcly action on (2R,1′S)-[1-1H2]FC yields an isoprenoid product with essentially no deuterium retention. On the other hand, the isoprenoid product of Pcly action on (2R,1′R)-[1-1H2]FC retains essentially all of the deuterium. These results indicate that the Pcly reaction is stereospecific and that the pro-S hydrogen of the FC substrate is transferred during the reaction.

Steady-state Kinetics and Isotope Effects—Initial steady-state kinetic analysis of Pcly revealed typical Michaelis-Menten behavior using FC as a substrate (Fig. 3). The kinetic parameters are summarized in Table II. Pcly is a very slow enzyme, with a kcat of 0.008 s−1. Whereas this turnover number is low compared with many other oxidases (34–39), it is similar to those of the mammalian CaaX prenyltransferases that attach the isoprenoid moiety to the Cys residue (40, 41). The Km value determined for FC using the recombinant enzyme (3 μM) is similar to that obtained for Pcly purified from native tissue (12). Kinetic parameters were also determined for Pcly action on the two deuterated isomers (Fig. 3; Table II). No Vmax isotope
Deuterium content of (E,E)-farnesols isolated from PCLase incubations with labeled FCs followed by NaBH₄ reduction

The deuterium content in the resulting farnesol was determined by GC-mass spectrometry analysis. The actual percentage of deuterium in the farnesol isomers was calculated by use of commercial (E,E) farnesol as standard. The variance is estimated to be about ±1-2% based on background intensity.

| Substrate | d₀ | d₁ |
|------------|----|----|
| (2R, 1'S)FC-[^4]H₁ | 98.8 | 1.2 |
| (2R, 1'R)FC-[^4]H₁ | 2.1 | 97.9 |

* The d₁ isotope descriptor denotes the presence of one deuterium atom in the predominant labeled form of the FC substrate (i.e. in the 1'S or 1'R position).

The action of Pcly on FC generates three products: cysteine, hydrogen peroxide, and farnesal (Fig. 1). We first examined cysteine and hydrogen peroxide as product inhibitors of Pcly. Surprisingly, cysteine did not inhibit Pcly up to the highest achievable concentration of 100 mM (data not shown), indicating a very weak affinity of this product for the enzyme. Whereas analysis of hydrogen peroxide inhibition can sometimes be complicated, since this oxidizing agent can irreversibly inhibit enzymes (44), this phenomenon did not appear to be the case for Pcly. In fact, hydrogen peroxide did not inhibit Pcly up to a concentration of 5 mM (data not shown), indicating very weak affinity for this product as well.

We then tested farnesal as a product inhibitor of Pcly. The experiment was performed with FC as the variable substrate and oxygen concentration fixed at air saturation in the presence of a range of farnesal concentrations. Analysis of the data via a double-reciprocal plot of 1/V₀ versus 1/[FC] (Fig. 4A) revealed a primarily noncompetitive inhibition pattern for farnesal versus FC. The data do not completely rule out a potentially more complex pattern, termed mixed inhibition, since the lines do not intersect exactly on the 1/[FC] axis. However, both noncompetitive and mixed inhibition patterns would clearly indicate that the binding sites of FC and farnesal do not overlap. Thus, farnesal does not bind to the same form of the enzyme as FC (i.e. the unliganded oxidized state).

The prenyl alcohol farnesol can be considered a dead end inhibitor of the Pcly reaction, since this compound is an analog for the product farnesal. Farnesol was examined for its inhibitory properties under conditions at which the oxygen concentration was fixed at air saturation, and FC concentration was varied. Analysis of the data via a double reciprocal plot of 1/V₀ versus 1/[FC] (Fig. 4B) demonstrated that farnesol is also a noncompetitive inhibitor versus FC. The noncompetitive inhibition pattern indicates that farnesol binds to the enzyme after the substrate FC binds. Thus, with farnesal, farnesol does not bind to the unliganded enzyme in the oxidized form.

Pre-steady-state Kinetics—The discovery of a steady-state isotope effect in the Pcly reaction suggested that hydride transfer is one of the slow steps in the Pcly reaction. To further elucidate the individual steps in its reaction cycle, we undertook a pre-steady-state analysis of the enzyme. Pcly was preincubated with the components needed for hydrogen peroxide detection, and then increasing concentrations of FC were added. As can be seen in Fig. 5A, a rapid burst of hydrogen peroxide production upon the addition of FC was detected. The amplitude of the observed bursts (i.e. the amount of hydrogen...
peroxide formed in this phase) was equal to the enzyme concentration ([H₂O₂]/[Pcly] = 1.2 ± 0.3), as would be expected if Pcly exhibited typical burst behavior. The rate constants for the exponential phase, kₐ₀, were obtained by fitting the data to a single exponential equation that contained a steady-state term (Equation 3); this analysis revealed a hyperbolic dependence of kₐ₀ on FC concentration (Fig. 5B). The maximum value of kₐ₀, denoted kₐ₀, was calculated according to Equation 4. The value of kₐ₀ is about 70-fold greater than the steady state turnover rate (0.62 s⁻¹ versus 0.008 s⁻¹) (Table III). This observation implies the hydride transfer step, which is a necessary component of kₐ₀, is not, in fact, even partially rate-limiting in the reaction. Further support for this conclusion comes from analysis of a similar pre-steady-state experiment using (2R,1'R)⁻[1⁻²H₁]FC as a substrate. This analysis, the results of which are also shown in Table III, indicated that there is no primary deuterium isotope effect on kₐ₀. The implications of this finding are discussed below.

The steady-state rate of hydrogen peroxide formation by Pcly correlates well with the rate of cysteine formation (14), indicating that the enzyme produces a stoichiometric amount of hydrogen peroxide during the reaction. To determine whether there was a burst of cysteine formation similar to that of hydrogen peroxide formation, we performed a pre-steady-state analysis of the formation of this product also (See “Experimental Procedures”). Since the steady-state rate of Pcly is so slow, we were able to perform the analysis by hand quenching at the times indicated (see legend to Fig. 6). However, only a linear increase in the rate of cysteine formation was observed, with no apparent rapid burst (Fig. 6). The rate of linear increase (0.0035 s⁻¹) is within a factor of 2 of the steady state kₐ₀ determined by measuring hydrogen peroxide formation (0.008 s⁻¹; Table II). This observation indicates that cysteine formation is one of the primary rate-determining steps in the Pcly reaction.

**DISCUSSION**

Whereas the biochemical mechanisms and biological consequences of protein prenylation have been established, little is known about the fate of prenylated proteins. Understanding the fate of the prenylcysteine moiety they contain, is of considerable importance, since free prenylcysteines can disrupt a variety of cellular signaling processes (8–11). Cellular degradation of prenylcysteines is thought to be primarily catalyzed by Pcly, a lysosomal thioether oxidase. In the present study, we conducted stereochemical and kinetic analyses of this enzyme to determine how it achieves this unusual type of catalysis.

Cleavage of the C–H bond at carbon-1 of the isoprenoid moiety during the Pcly reaction is supported by the deuterium isotope effect studies using the substrate (2R,1'S)⁻[1⁻²H₁]FC (Fig. 1). The studies demonstrating that the Pcly reaction is stereospecific, in which the pro-S hydride is transferred to FAD, also support this conclusion. Stereochemical removal of the pro-S hydrogen at C-1 of the farnesyl chain indicates tight binding of the FC substrate in the Pcly active site, in accord with the submicromolar apparent Kₘ values measured previously (0.69 µM for FC and 0.84 µM for geranylgeranylcysteine) (14). The small primary kinetic isotope effect (kₐ/kₐ₀ = 2) determined for degradation of FC bearing deuterium in the pro-S position under steady-state conditions as measured by H₂O₂ production appears to indicate at least one rate-determining hydrogen (deuterium) transfer in the overall mechanism. However, the absence of a detectable isotope effect on the pre-steady-state rate is inconsistent with the expectation of an initial rate-limiting cleavage of the isoprenoid C–H bond adjacent to sulfur. Noncompetitive inhibition kinetics with the product farnesal and with farnesol under steady-state conditions suggest that these very similar compounds do not bind to the free enzyme in its oxidized form. These new experimental findings provide important insights into the catalytic process.

The mechanisms of flavoprotein-catalyzed redox reactions vary widely and may involve free radicals, radical ion pairs, carbanions, carbocations, and covalent adducts with the heterocyclic cofactor (48, 49). In the case of Pcly, no evidence is presently available to distinguish a direct hydride transfer from FC to FAD in the oxidative half-reaction from more complex scenarios involving single-electron transfers and short lived radical pair intermediates such as those proposed for monoamine oxidase reactions (49, 50). However, the stereospecificity of hydrogen removal from C-1 of the farnesyl chain seems inconsistent with a conformationally unrestricted FC free radical intermediate. It seems more likely that the hydrophobic polyene chain is fixed in the active site with the pro-S hydrogen positioned near N-5 of the oxidized FAD cofactor (or an active site mediator) by analogy with UDP-N-acetylenolpyruvylglucosamine reductase (51). In addition, alignment of the C–H bond with the π-orbitals of the adjacent 2,3 double bond would stabilize the transition state for an initiating hydride (or hydrogen atom) transfer on the way to a planar conjugated thiocarbenium ion (or ω-thioallyl radical). In view of the precedent for hydride transfer mechanisms in stereospecific flavin monooxidase reactions (51, 52) and in the absence of contravening evidence, we opt for a minimal kinetic scheme portraying a hydride transfer oxidation (Fig. 1).

The experimental results outlined above can be accommodated by the six-step reaction pathway shown in Fig. 7. However, it seems inevitable that this minimal kinetic mechanism will be modified in the future to take into account new experimental findings. In the mechanism proposed, FC binding to the enzyme (step 1) is followed by hydride transfer from C-1 of the isoprenoid moiety of FC to FAD, forming reduced flavin and FC containing a sulfur-stabilized carboxylation (see first intermediate in Fig. 1). This thiocarbenium ion reacts rapidly with a water molecule to form the hemithioacetal intermediate (designated FC') (Fig. 7). Thus, in this case, the water molecule participates as a nucleophile as the hydride is being abstracted in step 2 of the kinetic mechanism or immediately afterward. The second substrate (molecular oxygen) now reacts with enzyme-FC', forming a transient ternary complex (not shown) that serves to reoxidize the flavin and generate hydrogen per-
Oxide. The hemithioacetal then collapses to enzyme-bound farnesal and cysteine, followed by an ordered release of products in which farnesal is liberated before cysteine.

The noncompetitive nature of both inhibitors in this study (farnesal and farnesol) demonstrates that neither compound inhibits Pcly by binding to the enzyme in its unliganded oxidized form (E-Floxid in Fig. 7). These hydrophobic compounds must therefore bind to the enzyme after FC, perhaps to an enzyme-bound intermediate. We propose that farnesal and farnesol exert their inhibitory effects by binding to the oxidized enzyme-cysteine binary complex (E-Floxid/Cys in Fig. 7) after the product farnesal is released.

The pre-steady-state data can also be explained by the proposed mechanism. The rapid burst of a stoichiometric amount of hydrogen peroxide, but not of cysteine, dictates that hydrogen peroxide must be formed and released prior to cysteine in the Pcly reaction, indicating that a ternary complex containing reduced enzyme, the hemithioacetal (FC −), and oxygen must be formed in step 3. The finding that the rate of cysteine formation is within 2-fold of the steady state turnover rate requires that cysteine production and/or one of the subsequent steps in the second half-reaction shown in Fig. 7 are rate-limiting in the reaction. Thus, in our sequential mechanism, the rapid “burst” of hydrogen peroxide formation is followed by slower steps.
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The time course of fluorescence can be described by a single exponential equation with a steady state term (Equation 3). The rate constant for the exponential phase is \( k_{\text{burst}} \).

### TABLE III

| Substrate | \( k_{\text{burst}} \) | \( K_{1/2} \) | \( [\text{H}_{2}\text{O}_{2}]/[E] \) |
|-----------|-----------------|--------------|------------------|
| FC        | 0.62 ± 0.02     | 1.6 ± 0.4    | 1.2 ± 0.3        |
| \((2R,1'S)\)−[1'−2\text{H}]FC | 0.60 ± 0.01    | 1.1 ± 0.12    | 1.1 ± 0.2        |

\( ^a \) The rate constants for \( k_{\text{burst}} \) display a hyperbolic dependence on FC concentration. Using Equation 4, the maximum value, \( k_{\text{burst, max}} \), can be determined.

\( ^b \) The hydrogen peroxide concentration is obtained from a standard resorufin curve, in which the amplitude of the burst in fluorescence units can be converted to a concentration of hydrogen peroxide produced.

By analogy, the isotope effect value of 2 determined for the Pcly reaction implies that another step (such as decomposition of the hemithioacetal intermediate, release of farnesal, and/or release of cysteine) must be the primary rate-limiting step.

The data presented here clearly show that the deuterium isotope effect occurs at a step after hydrogen peroxide production (step 3) and hence after hydride transfer. A plausible chemical mechanism can be formulated to explain this apparent anomaly. After abstraction by the flavin, the deuterium atom is transferred to an active site base that is very well sequestered in the active site such that no exchange with solvent can occur. This same base then functions as the general acid that catalyzes the breakdown of the hemithioacetal shown in Fig. 1. Hence, transfer of the deuterium results in an isotope effect that is manifest not on the first half-reaction that produces hydrogen peroxide but rather on the step that produces the cysteine and farnesal products. Whereas this explanation is logical, formal proof would require a demonstration that the deuterium atom is incorporated into the cysteine product. This will be quite difficult to demonstrate experimentally, since the cysteine thiol deuterium would exchange rapidly with solvent upon release of the product. However, precedent exists for kinetically sequestered hydrogens on other reduced flavin intermediates generated by hydride transfer (see Ref. 51 and references therein).

Whereas the model outlined in Fig. 7 is not the only type of mechanism that could be drawn for Pcly, we believe it is the simplest one consistent with all the available data. Although Pcly appears to be a unique enzyme in the reaction that it catalyzes (i.e. oxidative cleavage of a thioether bond with no net oxidation at sulfur), its mechanism shares some properties with other oxidases. Whereas it is more common for an oxidase to display a ping-pong kinetic mechanism (44, 47, 56, 57), a sequential mechanism, like the one proposed here for Pcly, is not unprecedented. For example, L-amino acid oxidase (58), urate oxidase (59), and D-amino acid oxidase (46) exhibit sequential kinetic mechanisms.

In summary, evidence is provided in support of a sequential kinetic mechanism for Pcly. More detailed pre-steady-state studies are likely to yield significant findings on how this novel enzyme achieves catalysis. Although the proposed hemithioacetal intermediate seems likely, in the absence of more direct evidence, its structure is hypothetical, and other possibilities (e.g. with farneslycysteine covalently attached to the enzyme or the flavin) cannot be excluded. In addition, substrate specificity studies will further our understanding of the Pcly active site. Equally important are ongoing efforts to understand the cellular role of Pcly in the metabolism of prenylcysteines (i.e. the determination of whether Pcly has an essential physiological function).
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