Substrate Specificity of Mammalian Prenyl Protein-Specific Endoprotease Activity†

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Received September 15, 1997

ABSTRACT: We have previously identified proteolytic activity in rat liver microsomes that cleaves an intact tripeptide, VIS, from *S*-farnesylated-CVIS tetrapeptide. This enzymatic activity, termed prenyl protein-specific endoprotease (PPEP) activity, has been solubilized in CHAPS and purified 5-fold. To probe the peptide recognition features of PPEP activity, 64 tripeptides [*N*-acetyl-C(*S*-farnesyl)a_1a_2] were prepared and tested as competitive inhibitors of PPEP activity-catalyzed hydrolysis of *N*-acetyl-C(*S*-farnesyl)VI-[^{3}H]S. It was found that PPEP activity prefers large hydrophobic residues in the a_1 and a_2 positions. A subset of *N*-acetyl-C(*S*-farnesyl)a_1a_2 peptides were prepared in radiolabeled form, and it was found that PPEP activity preferences for these substrates correlated well in most cases with the inhibition data. The exception is that R in the a_1 position does not prevent binding of peptide to PPEP activity, but such peptides are poor substrates. The anionic residue D in the a_2 position is not tolerated by PPEP activity. Five farnesylated radiolabeled tetrapeptides, Ac-C(F)FM[^{3}H]L, Ac-C(F)LI[^{3}H]L, Ac-C(F)LL[^{3}H]L, Ac-C(F)LM[^{3}H]L, and Ac-C(F)VI[^{3}H]L were prepared, and PPEP activity kinetic studies revealed that they are good substrates and show comparable K_M values (2.2−13.5 μM). Ac-C(F)RL[^{3}H]S is a poor substrate. The reported peptide binding preferences of PPEP activity should be useful in designing compounds that block the C-terminal proteolysis of prenylated proteins. Nonprenylated peptides do not bind to PPEP activity, and replacement of the farnesyl group with an *n*-pentadecyl group modestly reduces binding. Peptide−membrane partitioning studies were used together with theoretical arguments to fully understand the substrate specificity of PPEP activity toward these compounds.

A diverse group of eukaryotic cell proteins has been reported to be posttranslationally modified by prenylation (*1*, *2*). Such proteins include yeast a-mating factor (*3*), Ras proteins (*4*−*6*), other GTP-binding proteins (*7*−*9*), and nuclear lamins (*10*, *11*). All of these prenylated proteins contain a carboxyl-terminal CysAliAliXaa (referred to in this paper as Ca_1a_2X) sequence (Ali is usually but not necessarily an aliphatic amino acid, and Xaa can be a variety of amino acids). Prenylation occurs first on the cysteine residue via a thioether linkage with either a 15-carbon farnesyl or a 20-carbon geranylgeranyl group, followed by proteolytic removal of the last three amino acids. Finally, the biologically reversible *S*-adenosyl-methionine-dependent methylation of the newly exposed α-carboxyl group of the *S*-prenyl-cysteine completes the final modification, although some proteins are further modified by palmitoylation of nearby cysteine residues (*12*).

Protein farnesyltransferase (PFT) (*13*, *14*) and protein geranylgeranyltransferase-I (PGGT-1) (*15*, *16*) catalyze transfer of farnesyl or geranylgeranyl from farnesyl pyrophosphate or geranylgeranyl pyrophosphate, respectively, to Ca_1a_2X-containing proteins and have been identified and purified from cytosol fractions of mammalian and yeast cells (*17*). The type of prenyl group added to the protein is dictated primarily by the X residue (*18*−*24*). PFT preferentially farnesylates Ca_1a_2X-containing proteins in which X is serine, methionine, glutamine, cysteine, or possibly other residues. PGGT-1 preferentially geranylgeranylates proteins having a carboxyl-terminal leucine or phenylalanine.

The prenyl protein-specific methyltransferase (*25*−*28*) and methylesterase (*29*) have been detected in the membrane fraction of mammalian tissue. Genetic studies in yeast demonstrate that both farnesylated and geranylgeranylated cysteine residues are methylated by the same enzyme (*30*, *31*). The gene *STE14* encodes the prenyl protein-specific methyltransferase in yeast. The nucleotide sequence reveals that the enzyme encoded by it contains 239 amino acids and multiple transmembrane domains (*32*, *33*). Although the mammalian counterpart of this enzyme has not yet been purified, its characterization (*34*−*36*) and kinetic mechanism of the action (*37*) have been studied in its membrane-bound state.

The prenyl protein-specific endoprotease (PPEP)[1] activity has been detected in homogenates from yeast (*38*) and bovine (*39*) and rat (*40*) liver membranes with synthetic farnesylated and geranylgeranylated peptides as substrates. In all cases, a single endoproteolytic event occurs to release an intact

† This work was supported by grants from the National Institutes of Health (CA52874 and Research Career Development Award GM562) to M.H.G.

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[1] Abbreviations: Ac-C(F)a_1a_2 and Ac-C(F)a_1a_2X, tri- and tetrapeptides containing *N*-acetyl-L-(*S*-farnesyl)-cysteine linked to dipeptides a_1a_2 or tripeptides a_1a_2X, respectively; [^{3}H]Ac-C(F)a_1a_2, tritium is on the acetyl group; ECB-C(F)VI[^{3}H]S, C(F)VI[^{3}H]S tetrapeptide with a biotin-CONH$(CH_2)_5$CO attached to the N-terminus ([^{3}H]S, 3-[^{3}H]-L-serine); [^{3}H]L, 4, 5-[^{3}H]-L-leucine; PPEP activity, mammalian prenyl protein-specific endoprotease activity that cleaves the C-terminal tripeptide from prenylated proteins.

S0006-2960(97)02289-7 CCC: $15.00
Published on Web 03/06/1998

tripeptide (a_1a_2X) from the structure C(*S*-prenyl)a_1a_2X, and proteolysis occurs only if the peptide is prenylated. Cell fractionation studies suggest that PPEP activity is mainly localized in the endoplasmic reticulum (*40*). PPEP activity can cleave prenylated tri- and tetrapeptides with the structure *N*-acetyl-C(*S*-prenyl)a_1a_2 or *N*-acetyl-C(*S*-prenyl)a_1a_2X (*39*, *40*). Peptides with these structures but containing D-amino acids in place of L-C, L-a_1, or L-a_2 are not PPEP activity substrates (*41*). PPEP activity is not inhibited by a variety of standard protease inhibitors but is sensitive to the thiol reagent *p*-chloromercuribenzoate (*39*, *40*). Analogues of short prenylated peptides containing protease-resistant functional groups in place of the protease-susceptible amide were prepared, and some were found to be potent inhibitors of PPEP activity (*42*). PPEP activity has been solubilized and partially purified (~10-fold) from bovine liver microsomes (*43*) and appears to be in an aggregate form (MW ~ 641 kDa, estimated by gel filtration). The partially purified enzyme is unstable, and its purification to homogeneity has not been reported.

More recently, two genes (*RCE1* and *AFC1*) responsible for PPEP activity were identified in yeast by genetic methods (*44*). The sequences of Rce1p and Afc1p, which these genes encode, indicate that they are integral membrane proteins. The yeast mutant containing defects in both genes loses the ability to proteolytically process prenylated proteins. Loss of proteolysis renders Ras2p, which normally localizes to the plasma membrane, to mislocalize to internal membranes, at least when overexpressed. Furthermore, loss of proteolysis reduces but does not eliminate Ras2p function in yeast expressing either high or endogenous levels of this protein. It has been shown that proteolysis and methylation are important in promoting efficient membrane binding and function of K-Ras4B (*45*). In *Xenopus* oocytes, proteolysis and methylation are probably required for palmitoylation, membrane binding, and function of H-Ras (*46*). These studies suggest that PPEP activity and the methyltransferase, like PFT, may be good targets for anti-oncogenic therapeutics, especially because yeast lacking PPEP activity are viable (*44*).

In the present study, membrane-bound PPEP activity was solubilized from rat liver microsomes using CHAPS and was partially purified. Studies of the binding of a large number of peptides of the type *N*-acetyl-C(*S*-farnesyl)a_1a_2 [Ac-C(F)-a_1a_2] to partially purified PPEP activity revealed that the enzyme prefers aliphatic or aromatic amino acids at the a_1 and a_2 positions. A series of radiolabeled peptides of the type [^{3}H]Ac-C(F)a_1a_2 were also prepared and tested as substrates of partially purified PPEP activity, and the results are consistent with those obtained from binding studies. Furthermore, six farnesylated tetrapeptides were prepared, and kinetic studies revealed that the presence of arginine in the a_1 position prevents proteolysis but not peptide binding to PPEP activity. PPEP activity is not inhibited by various nonprenylated Ca_1a_2X-containing peptides at concentrations 17-fold higher than that of the prenylated substrate, and replacement of the farnesyl group by an *n*-pentadecyl chain modestly reduces PPEP activity-peptide binding.

MATERIALS AND METHODS

Peptide Syntheses. Full details for the syntheses of all peptides used in this study are given as Supporting Information. The general strategies are briefly mentioned here. Peptides of the type Ac-C(F)a_1a_2 or Ac-C(F)a_1a_2X were prepared by coupling dipeptide a_1a_2 or tripeptide a_1a_2X (prepared by standard solid-phase or solution synthesis or purchased commercially) with the *N*-hydroxysuccinimide ester of Ac-C(F). A few peptides were prepared by farnesylation of Ac-Ca_1a_2 or Ac-Ca_1a_2X. Final products were purified by HPLC and analyzed by ^{1}H NMR. Small amounts of the undesired diastereomer [D-configuration of the C(F) residue] were obtained in some cases, and these were removed from the desired all-L peptide by HPLC.

Prior to the synthesis of all radiolabeled peptides, the nonradiolabeled versions were synthesized, HPLC purified, and characterized by ^{1}H NMR. HPLC retention times provided a guide for the purification of radiolabeled peptides. Two methods were developed for the syntheses of [^{3}H]Ac-C(F), and this material was elaborated into tripeptides using the same methods that were used for the nonradiolabeled peptides. Peptides of the type Ac-C(F)a_1a_2[^{3}H]X were prepared by coupling Ac-C(F)a_1a_2 to [^{3}H]X-CO_2Me using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxybenzotriazole. The methyl ester was removed by saponification. Saponification using the specific conditions (Supporting Information) did not result in epimerization of the X residue as revealed by HPLC and ^{1}H NMR analysis of reactions carried out with nonradiolabeled peptides. All radiolabeled peptides have a specific activity of 0.74−1.0 Ci/mmol. To minimize radio decomposition, all peptides were stored in DMF or DMSO/H_2O (4/1) at typically 175 μCi/mL at −20 °C. Rapid decomposition occurs if peptides are stored as a dry film. When necessary, radiolabeled peptides were repurified by HPLC and used immediately in PPEP activity studies. Peptides containing an *S*-*n*-pentadecyl-C residue were prepared by treating the desired cysteine-containing peptide with *n*-pentadecyl bromide (Supporting Information).

The concentrations of all peptides in stock solutions were determined either by weighing the solid peptide (>1 mg amounts) or by integrating NMR resonances of stock solutions in DMSO-d_6 containing a known amount of CH_2Cl_2 as internal standard (NMR recycle delay set to 10 s). Solubilization and partial purification of PPEP activity from rat liver microsomes are given in full detail as Supporting Information.

PPEP Activity Assays. (i) Assays with Rat Liver Microsomes. The assay mixture (50 μL) contained 47.5 μL of buffer A, 1.5 μL (29 μg of protein) of a suspension of washed rat liver microsomes (Supporting Information), and 1 μL of Ac-C(F)VI[^{3}H]S or Ac-C(F)RL[^{3}H]S stock in DMF (final concentration 3 μM in the assay, added last). The reaction was incubated at 37 °C for 20 min. The reaction was stopped by placing the tube in boiling water for 6 min, and 150 μL of water, 1.6 μL of 7.2% CHAPS in buffer A (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 1 mM EGTA), 7 μL of buffer D (20 mM Tris-HCl, pH 7.2, 0.2% CHAPS, 2 M NaCl), and nonradiolabeled standard [VIS (5 μg) or RLS (10 μg)], were added. Precipitated protein was removed in a microfuge, and the supernatant was injected onto an HPLC column (Vydac 218TP104). The products were eluted with an isocratic solvent mixture of 97% solvent A (100% water/0.06% trifluoroacetic acid)/3% solvent B (100% acetonitrile/0.06% trifluoroacetic acid) at a flow rate of 1 mL/min (HPLC

method i). Serine elutes in the solvent front, and the retention times for LS, RLS, IS, and VIS are 5, 7.3, 4.8, and 9.7 min, respectively. For quantifying the amounts of peptide products, the eluants at 2.8−4.2 (S), 4.2−6.2 (IS or LS), 6.2−8.7 (RLS), and 8.7−11.2 min (VIS) were collected, and a 0.7 mL aliquot of each was mixed with 6 mL of scintillation cocktail for scintillation counting. Some microsomal assays were carried out in the presence of various concentrations of competing, nonradiolabeled farnesylated peptides (see Results and Discussion). In these assays, 3 μM Ac-C(F)-VI[^{3}H]S and 19 μg of microsomal protein were used.

(ii) Assays with Q Sepharaose Partially Purified PPEP Activity in the Presence of Peptide Inhibitors. A second isocratic solvent mixture (87% solvent A/13% solvent B at a flow rate of 1 mL/min, HPLC method ii) was used to elute the major peptide product, VI[^{3}H]S [2.8−5.6 min, using Ac-C(F)VI[^{3}H]S as substrate], from the HPLC column. This rapid HPLC method was used to assay PPEP activity after gel filtration or Q Sepharose chromatography, since after these steps little, if any, [^{3}H]S- and I[^{3}H]S-producing enzymes were detected. The assay mixture (50 μL) contained 22−23 μL of buffer (20 mM Tris-HCl, pH 7, 0.2% CHAPS) plus peptide inhibitor delivered in 1−2 μL of DMF, DMSO/water (4/1), or DMSO-d_6, 1 μL of substrate stock of Ac-C(F)VI[^{3}H]S in DMF (final concentration 3.5 μM in the assay), and 25 μL of Q Sepharaose partially purified PPEP activity (4.5 μg) added last to initiate the reaction. The reaction was incubated at 37 °C for 40 min. The reaction was stopped by placing the tube in boiling water for 6 min, and 150 μL of water was added. After centrifugation in a microfuge, the supernatant was injected onto the HPLC column. The product was quantified as described above.

(iii) Assays with Q Sepharaose Partially Purified PPEP Activity and [^{3}H]Ac-C(F)a_1a_2. Q Sepharose partially purified PPEP activity (10 μg, 39 μL) was incubated with 1 μL of substrate stocks of the peptides [^{3}H]Ac-C(F)a_1a_2 (1 Ci/mmol) in DMF at 37 °C for 90 min. For [^{3}H]Ac-C(F)a_1a_2 peptides of lower specific activity (0.74 Ci/mmol), Q Sepharose partially purified PPEP activity (8 μg, 58 μL) was incubated with 2 μL of substrate stock in DMSO/water (4/1) at 37 °C for 90 min. Both reactions were quenched with 60 μL of 90% methanol containing 0.2 M acetic acid, the reaction mixture was transferred to a 0.5 mL tube to which 2−5 μg of each of the unlabeled standard, Ac-C(F), and the corresponding Ac-C(F)a_1a_2 peptide were added. The original tube was rinsed twice with 30 μL portions of DMF, and the reaction mixture and the rinse were combined and injected onto the HPLC column with gradient H (80% solvent A/20% solvent B to 65% B in 40 min, to 80% B in 10 min, and to 100% B in 10 min) at a flow rate of 1 mL/min. The common product, [^{3}H]Ac-C(F), coeluting with the unlabeled standard at 38.4 min was collected (37.3−39.3 min), and 0.6 mL of the eluant was mixed with 7 mL of scintillation fluid for scintillation counting. To further confirm the identity of the product, the product fraction was dried down in a Speed-Vac (Savant Instruments), and the residue was dissolved in a small volume of methanol and spotted onto a TLC plate (Merck silica gel, F_{254}, 0.25 mm, 20 × 20 cm). The plate was developed with chloroform:methanol:32% aqueous acetic acid (6:2:0.2 by volume). After being dried, the plate was exposed to iodine to reveal the unlabeled standard [Ac-C(F), which was included in the HPLC run]. After iodine was evaporated, fluorography of the TLC plate was carried out by spraying it with EN^{3}HANCE (NEN), wrapping it with Saran Wrap, and placing it in a film cassette with an intensifying screen (Kodak X-Omatic) and X-ray film (Kodak X-OMAT AR) at −80 °C for 48−96 h.

(iv) Measurement of V_{max} and K_M of Ac-C(F)a_1a_2[^{3}H]X Substrates with Q Sepharaose Partially Purified PPEP Activity. Q Sepharose partially purified PPEP activity (7.7 μg, 50 μL) was incubated with 1 μL of different amounts of substrate stocks of Ac-C(F)a_1a_2[^{3}H]X (Table 3) in DMF at 37 °C for 60 min. The reaction was stopped by placing the tube in boiling water for 6 min, and 150 μL of water and 5−10 μg of the corresponding unlabeled a_2X and a_1a_2X products were added. After centrifugation in a microfuge, the supernatant was analyzed by HPLC. For Ac-C(F)VI-[^{3}H]S, HPLC method i was used, and the amount of VI-[^{3}H]S was quantified as the above. For Ac-C(F)VI[^{3}H]L, an isocratic solvent mixture of 86% solvent A/14% solvent B at a flow rate of 1 mL/min was used (HPLC method iii). Leucine elutes in the solvent front, and the retention times for IL and VIL are 7.2 and 13.6 min, respectively. For quantifying the amount of VI[^{3}H]L, the eluant at 12.5−15.5 (VIL) was collected, and an aliquot (0.7 mL) of this fraction was mixed with 6 mL of scintillation cocktail for scintillation counting. For the other peptide substrates (Table 3), an isocratic solvent mixture of 81% solvent A/19% solvent B at a flow rate of 1 mL/min was used (HPLC method iv). Leucine elutes in the solvent front, and the retention times (min) for di- and tripeptides are as follows: IL (5), LL (5.5), ML (4.8), FML (16.6), LIL (11.6), LLL (13.7), and LML (10.8). For quantifying the amounts of the tripeptide products, the eluants at 15.7−18.7 min (FML), 10.2−13 min (LIL), 12.2−15 min (LLL), and 9.4−12 min (LML) were collected, and an aliquot (0.7 mL) of each fraction was mixed with 6 mL of scintillation cocktail for scintillation counting.

Membrane Binding and Kinetic Studies with the Microsomal Membranes. The experiment was set up exactly as for the microsomal PPEP activity assay but with 40 μM competing, nonradiolabeled farnesylated peptide in the absence of radiolabeled substrate. The nonradiolabeled farnesylated peptide (14 μL of a 2 mM stock in DMSO-d_6) and 14 μL of DMF were added to a polyallomer microfuge tube (1.5 mL, Beckman). To the tube, 658 μL of buffer A and 14 μL of microsomes (19 mg of protein/mL) were added, and the tube was briefly vortexed and incubated at 37 °C for 20 min. A control experiment was carried out in parallel except that 14 μL of buffer A was added instead of microsomes. Samples were ultracentrifuged at 25 °C at 96900g_{max} for 70 min in KA-30 rotor (Composite Rotor, Mountain View, CA). The supernatant of each sample was carefully removed and mixed with 350 μL of 90% methanol containing 0.2 M acetic acid, and the resulting mixture was dried in a Speed-Vac. For the control sample, 350 μL of 90% methanol containing 0.2 M acetic acid were added directly into the tube and dried in a Speed-Vac. The dried residue and the pelleted microsomes were separately dissolved in 70 μL of DMF (sonication for 1−2 min followed by 1 min vigorous vortexing), and samples were microfuged for 2 min to pellet the insoluble materials. After removal of the supernatant to a new polyallomer microfuge tube, 15 μL of DMF and 15 μL of water were added to the original tube and proceeded as above. The supernatants were com-

bined and 10 μL of 2 mM internal standard was added [using Ac-C(F)VI for Ac-C(F)ID, Ac-C(F)IQ, and Ac-C(F)RL; using Ac-C(F)ST for Ac-C(F)IV and Ac-C(F)VI]. The mixture was injected onto the HPLC column with gradient H at a flow rate of 1 mL/min. The computer-digitized peptide peaks were integrated.

RESULTS AND DISCUSSION

In our early studies, we used rat liver microsomes as a crude source of PPEP activity and the biotinylated, farnesylayed, and radiolabeled peptide ECB-C(F)VI[^{3}H]S (patterned after the C-terminus of the γ subunit of transducin) as PPEP activity substrate (*40*). On the basis of detailed kinetic analyses, we were able to establish the source of the proteolytic products, [^{3}H]S, I[^{3}H]S, and VI[^{3}H]S. We concluded that a VI[^{3}H]S-producing enzyme activity (PPEP activity) is present that did not utilize nonfarnesylated peptides. We also concluded that I[^{3}H]S is derived solely by proteolysis of VI[^{3}H]S and that production of [^{3}H]S comes from ECB-C(F)VI[^{3}H]S and I[^{3}H]S; both reactions being catalyzed by proteases in crude microsomes that are presumably not part of the prenyl protein proteolysis pathway because they showed no requirement for the peptide farnesyl group.

Solubilization and Partial Purification of PPEP Activity. As described in Supporting Information, we have now been able to detergent-solubilize PPEP activity from microsomes using 0.5% CHAPS. The CHAPS concentration in PPEP activity assays is typically 0.2%, and higher concentrations lead to progressive inhibition. Thus, subsequent column chromatographies of solubilized PPEP activity were carried out with buffer containing 0.2% CHAPS so that the minimally diluted column fraction could be used in the assay mixture (needed because of the low abundance of PPEP activity in tissue).

The post-CHAPS solubilized (126000g_{max} supernatant) and filtered (0.22 μm) PPEP activity was first analyzed by gel filtration on a calibrated Superdex 200HR 10/30 FPLC column (Pharmacia, 23.6 mL) using elution buffer with two different concentrations of CHAPS, 0.2% (3.2 mM) and 0.5% (8.1 mM). The cmc of CHAPS at the ionic strength that we are using for the gel filtration is 6−10 mM. In both cases, the activity peak eluted in the void volume along with the marker blue dextran 2000 (not shown). With 0.5% CHAPS, the recovery of PPEP activity was only 10%, but the recovery in the presence of 0.2% CHAPS was ∼110%. After completion of this phase of our study, Chen and coworkers reported that PPEP activity can be solubilized from bovine liver microsomes in the presence of 1% CHAPSO and that the enzymatic activity eluted in the void volume of a Sephadex 200 HR column (*43*). These consistent results suggest that even under optimized solubilization conditions, based on yield of enzymatic activity, PPEP activity seems to form large aggregates with itself or other proteins when chromatographed with 0.2% CHAPS; the possibility of large aggregates of protein with CHAPS micelles cannot be ruled out. As described in Supporting Information, in the presence of 0.2% CHAPS, PPEP activity can be partially purified by gel filtration (∼5-fold, Figure 1) on a calibrated Superdex 200HR 16/50 FPLC column and anion exchange chromatography (∼4-fold, major peak in Figure 2). These preparations produce virtually only VI[^{3}H]S from ECB-C(F)VI[^{3}H]S and Ac-C(F)VI[^{3}H]S, and the results also confirm our earlier studies showing that I[^{3}H]S and [^{3}H]S are produced by proteases in microsomes distinct from PPEP activity. The pH−rate profiles for PPEP activity partially purified by gel filtration and anion exchange were very similar with a pH peak optimum around 6−6.5 (not shown). Table 1 summarizes the solubilization and partial purification of PPEP activity through the hydroxylapatite step (see Supporting Information).

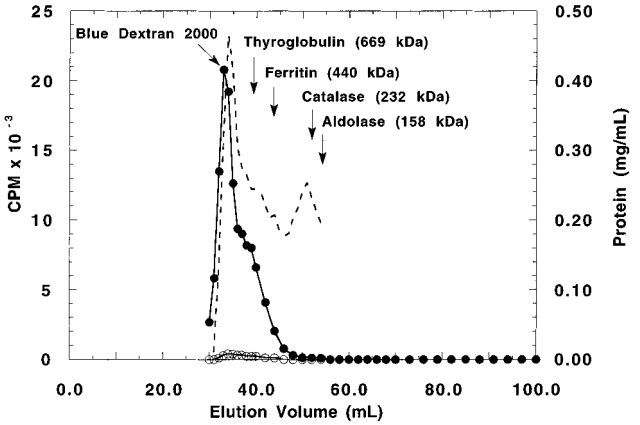

FIGURE 1: Chromatography of 0.5% CHAPS-solubilized PPEP activity on a calibrated Superdex 200 HR 16/50 column. The solubilization of rat liver microsomes and the operation of the column are described under Supporting Information. Fractions of 1 mL (up to 70 mL) and of 1.5 mL (71−100 mL) were collected. Aliquots (10 μL) of each fraction (30−40) and every other fraction (42−84) were assayed (50 μL assay volume) for PPEP activity (closed circles VI[^{3}H]S, open circles I[^{3}H]S) with Ac-C(F)VI[^{3}H]S (1 Ci/mmol, 3.5 μM) as substrate (37 °C for 25 min). Products were analyzed by HPLC method i. Aliquots (10 μL) of each fraction (30−54) were assayed for protein (dashed line, BCA method with BSA as standard).

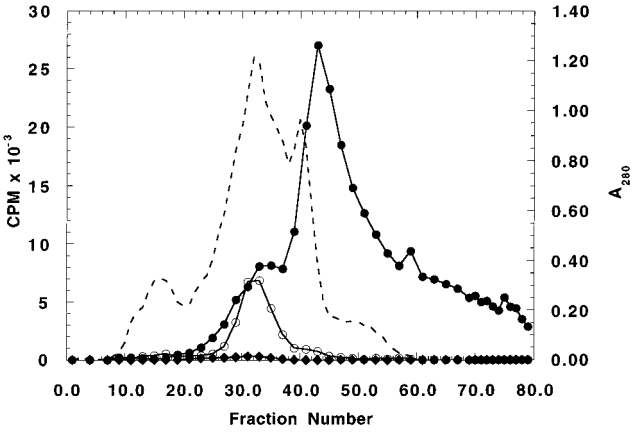

FIGURE 2: Chromatography of 0.5% CHAPS-solubilized PPEP activity on Q Sepharose Fast Flow. The gradient and the operation of the column are described under Supporting Information. Fractions were collected as follows: 1−24, 18 mL/fraction; 25−69, 14 mL/fraction; 70−79, 25 mL/fraction. Aliquots (50 μL) of every other fraction were assayed for PPEP activity (closed circles VI[^{3}H]S, open circles I[^{3}H]S, and closed diamonds for [^{3}H]S) with Ac-C(F)-VI[^{3}H]S (1 Ci/mmol, 3.5 μM) as substrate (37 °C for 25 min). Products were analyzed by HPLC method i.

Selection of Substrates for Specificity Studies. The major goal of the present study is to explore the substrate specificity of PPEP activity. We focused on the three amino acids on the C-terminal side of the *S*-farnesyl-cysteine residue;

Table 1: Partial Purification of PPEP Activity from Rat Liver Microsomes

fraction	protein (mg)	total activity (μunit)[a]			specific activity (μunit/mg)			purification (fold)			yield (%)		
		[^{3}H]S	I[^{3}H]S	VI[^{3}H]S	[^{3}H]S	I[^{3}H]S	VI[^{3}H]S	[^{3}H]S	I[^{3}H]S	VI[^{3}H]S	[^{3}H]S	I[^{3}H]S	VI[^{3}H]S
microsomes (34 mL suspension)	597	1015	9552	38208	1.7	16	64	1	1	1	100	100	100
0.5% CHAPS extract (78 mL)	296	296	4588	15510	1.0	15.5	52.4	0.59	0.97	0.82	29	48	40.6
Q Sepharose (fractions 43−52, 140 mL)	13.3	0	51	3312	0	3.8	249	0	0.2	3.9	0	0.5	8.7
hydroxylapatite concn (3.4 mL)[b]	7.4	ND[c]	ND	2218	ND	ND	300	ND	ND	4.7	ND	ND	5.8

[a] All assays were carried out with Ac-C(F)VI[^{3}H]S (1 Ci/mmol) using the various assay conditions given in Supporting Information for the various PPEP preparations. One μunit is defined as picomoles of product produced per min. [b] These data were based on using 50 mL of Q Sepharose fractions in the experiment (Supporting Information). [c] ND, not detectable.

presumably every substrate for PPEP activity will have a three amino extension beyond the prenylated cysteine. Since there are 20^3 possible tripeptide combinations, a full analysis of PPEP activity specificity is virtually impossible with limited amounts of enzyme. Rando and co-workers reported studies with PPEP activity and a series of substrates Ac-C(F)VIX, where X is varied (*41*). V_{max}/K_M for the tetrapeptide Ac-C(F)VIM is only 6.7-fold larger than V_{max}/K_M for the tripeptide N-Ac-C(F)VI. Der and co-workers studied the posttranslational modification of recombinant mutant Ras proteins in mammalian cells and found that when X = M, D, C, G, Y, S, N, T, Q, L, or F, the protein underwent farnesylation, proteolysis, and methylation (*47*). Previously, we found that the tripeptide ECB-C(F)VI at 20 μM causes 50% inhibition of the proteolysis of the tetrapeptide ECB-C(F)VI[^{3}H]S present in the assay at 2 μM (*40*). This point was further explored in the present study. With Q Sepharose purified PPEP activity, V_{max} and K_M for Ac-C(F)VI[^{3}H]S are 286 pmol min^{-1} mg^{-1} and 6 μM, respectively, and the values for [^{3}H]Ac-C(F)VI are 50 pmol min^{-1} mg^{-1} and 8 μM, respectively. Thus, the ratio of V_{max}/K_M for the tetrapeptide versus tripeptide is 7.6. Some additional studies with tetrapeptides versus tripeptides are described below. All of these results argue that, although PPEP activity "senses" the C-terminal residue, this residue is not critical for recognition by PPEP activity. On the basis of these findings, we decided to focus on the series of 64 tripeptides Ac-C(F)a_1a_2, where a_1 and a_2 are different amino acids (listed in Table 2).

Preparation of Ac-C(F)a_1a_2 and PPEP Activity Inhibition Analysis. As a general approach to preparing the series Ac-C(F)a_1a_2, a large amount of the N-hydroxysuccinimde ester of Ac-C(F) was prepared and distributed into a series of reaction tubes each containing one of the 64 a_1a_2 dipeptides (Table 2). Each of the 64 Ac-C(F)a_1a_2 tripeptides was purified by HPLC, and structures were confirmed by ^{1}H NMR. Each Ac-C(F)a_1a_2 tripeptide was tested for its ability to inhibit the PPEP activity-catalyzed production of VI[^{3}H]S from Ac-C(F)VI[^{3}H]S. It was first established that Ac-C(F)-VI, derived from the known PPEP activity substrate Ac-C(F)-VIS, inhibits the hydrolysis of 3.5 μM Ac-C(F)VI[^{3}H]S with an IC_{50} of 13 μM (not shown). Therefore, we decided to tested all Ac-C(F)a_1a_2 tripeptides at a concentration somewhat higher than 13 μM. All reaction mixtures contained PPEP activity that had been solubilized in CHAPS and purified on a Q Sepharose column, 3.5 μM Ac-C(F)VI[^{3}H]S, and 40 μM Ac-C(F)a_1a_2 test peptide. After a 40 min incubation at 37 °C, the reaction mixture was submitted to rapid, isocratic HPLC analysis of VI[^{3}H]S. In all cases, less than 5−10% of the total substrate was hydrolyzed, and thus the concentration of substrate during the assay remained close to its initial value. Inhibition data are summarized in Table 2 for all 64 Ac-C(F)a_1a_2 tripeptide competitors.

The following trends are apparent from the data in Table 2. When a_1 is a large hydrophobic residue (F, I, or L), potent inhibition is seen when a_2 is hydrophobic or somewhat hydrophilic (T), less inhibition is seen when a_2 is more hydrophilic (Q or S), and little if any inhibition is seen when a_2 is anionic (D). The same is true when a_2 is a large hydrophobic residue (L, M, V, F) in that good inhibition is seen even when a_1 is somewhat hydrophilic (C, N, Q, S, T). When both a_1 and a_2 are small hydrophobic residues (AA and AG), inhibition is modest. When both a_1 and a_2 are hydrophilic, uncharged residues (CS, HS, QN, SN, SS, ST), poor inhibition is seen. D in the a_2 position always leads to poor inhibition regardless of what is in the a_1 position. When a_1a_2 is CG, SA, SG, SP, and TG, i.e., a hydrophilic uncharged residue along with a small hydrophobic residue, weak inhibition is seen. W and Y are tolerated in the a_1 position; when a_1a_2 is WP, WQ, YL, YP, and YT, intermediate inhibition is seen. Interestingly, even K and R are tolerated in the a_1 position when a_2 is a large hydrophobic residue, and those peptides with R at a_1 bind somewhat better than those with K at a_1. Poor inhibition is seen when a_1 is K or R and a_2 is a small hydrophobic residues or a hydrophilic residue. *A clear conclusion of this extensive study is that those Ac-C(F)a_1a_2 tripeptides that have the most hydrophobic a_1a_2 dipeptide unit bind tightest to PPEP activity.* This result is in agreement with studies of the processing of K-Ras4B in transfected mammalian cells, where it was shown that proteins with VI following the farnesylated cysteine are efficiently proteolyzed (*47*). However, it is difficult to understand why the Ras protein with VY appears by SDS−PAGE gel shift analysis to be proteolyzed poorly (*47*).

Although we have studied mostly tripeptides, one should not automatically assume that the addition of the C-terminal X will not affect PPEP activity specificity. However, our tripeptide Ac-C(F)a_1a_2 inhibition results containing a variety of amino acids with respect to hydrophobicity and charge in the a_1 and a_2 positions still provide very useful information about PPEP activity specificity that can be utilized, for example, in the design of PPEP activity inhibitors.

Molecular Analysis of Ac-C(F)VIS as a PPEP Activity Inhibitor. Figure 3 shows the inhibition of PPEP activity-catalyzed hydrolysis of Ac-C(F)VI[^{3}H]S by a series of truncated Ac-C(F)VIS analogues, nonfarnesylated (Ac-CLML), and three farnesylated, full-length peptides. As expected, Ac-CLML shows very little, if any, inhibition (~8%) at the highest concentration tested (50 μM, 16-fold higher than the substrate concentration, 3.5 μM). Ac-C(F) and Ac-C(F)V are poor binders; Ac-C(F)VI shows interme-

Table 2: Percent Inhibition of PPEP Activity-Catalyzed Hydrolysis of 3.5 μM Ac-C(F)VIS[^{3}H]S by 40 μM Ac-C(F)a_1a_2

a_1 & a_2 large hydrophobic		a_1 mixed		
		a_2 anionic		
FL	86	HD	8	
FM	82	KD	13	α-25%, ε-75%[1]
FV	84	ID	5	
II	83	LD	9	
IM	82	ND	17	
IV	86	QD	10	
LI	83	RD	17	
LL	84	SD	9	
LM	84			
YL	69	a_1 & a_2 hydrophilic		
		CS	35	
a_1 small hydrophobic		HS	25	
a_2 large hydrophobic		QN	18	
AL	48	SN	12	
		SS	13	
a_1 large hydrophobic		ST	22	
a_2 small hydrophobic				
FA	80	a_1 & a_2 small hydrophobic		
WP	57	AA	24	
YP	48	AG	18	
a_1 hydrophilic		a_1 hydrophilic		
a_2 large hydrophobic		a_2 small hydrophobic		
CL	78	CG	48	
CM	56	SA	23	
CV	52	SG	21	
NI	40	SP	20	
NL	47	TG	26	
QI	51			
QL	59	a_1 cationic		
SF	57	a_2 mixed		
SI	53	KA	30	α-90%, ε-10%
SL	53	KF	34	α-40%, ε-60%
SV	32	KI	45	α-90%, ε-10%
TI	57	KM	25	α-70%, ε-30%
TV	37	KS	17	α-90%, ε-10%
		KV	30	α-70%, ε-30%
a_1 large hydrophobic		RI	72	
a_2 hydrophilic		RL	72	
FT	71	RR	42	
IQ	33	RV	60	
LS	60			
WQ	55	a_1 hydrophilic		
YT	38	a_2 cationic		
		TR	29	

[a] Ac-C(F)a_1a_2 peptides containing lysine are the indicated mixture of isomers (determined by ^{1}H NMR) in which the Ac-C−C(F) unit is attached to the α-NH_2 group or ε-NH_2 group of the dipeptide (Designated in table as [1].)

diate binding; and Ac-C(F)VIS, Ac-C(F)VIL, and Ac-C(F)-LML are the best binders. Thus, PPEP activity is sensitive to the length of the C-terminal peptide extension adjacent to the farnesylated cysteine. Consistent with the K_M results from Table 3 (described below) Ac-C(F)LML is the best

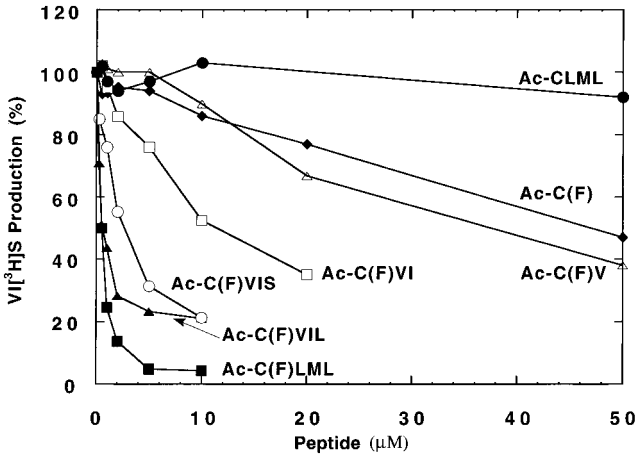

FIGURE 3: Inhibition of PPEP activity-catalyzed hydrolysis of Ac-C(F)VI[^{3}H]S (1 Ci/mmol, 3.5 μM) by nonradiolabeled peptides. The reaction was performed at the optimal pH of PPEP activity (pH 6). The assay mixture contained 28 μL of 100 mM sodium phosphate, 0.2% CHAPS (pH 6), 1 μL of various concentrations of peptide inhibitor stock in DMF or DMSO-d_6, 1 μL of substrate stock in DMF, and 20 μL of Q sepharose partially purified PPEP activity (3.2 μg), added last to initiate the reaction. Reaction mixture with Ac-CLML also contained 0.5 mm dithiothreitol. The reaction was incubated at 37 °C for 25 min. Assays were analyzed by HPLC method ii. The percent VI[^{3}H]S produced in the presence of the indicated concentration of the peptide inhibitor as compared to that produced in the absence of this inhibitor is plotted.

Table 3: PPEP Activity-Catalyzed Hydrolysis of 6−8.5 μM [^{3}H]Ac-C(F)a_1a_2 and K_M and V_{max} for Ac-C(F)a_1a_2[^{3}H]X (1 Ci/mmol) Substrates

a_1a_2 (sp act, Ci/mmol)	concn (μM)	[^{3}H]Ac-C(F) produced from [^{3}H]Ac-C(F)a_1a_2 vs [^{3}H]Ac-C(F)VI (%)
VI (1)	6	100
FM (1)	6	51
ID (1)	6	0
IM (1)	6	47
IQ (1)	6	5
IV (1)	6	45
LI (1)	6	50
LL (1)	6	83
LM (1)	6	120
VI (0.74)	8.5	100 (33)[a]
FL (0.74)	8.5	72 (34)
RL (0.74)	8.5	6 (24)
ST (0.74)	8.5	4 (4)

a_1a_2X	K_M (μM)	V_{max} (pmol min^{-1} mg^{-1})
VIS	6	287
VIL	5	426
FML	13.5	424
LIL	4.2	301
LLL	3.6	299
LML	2.2	170
RLS	not determined	very weak activity[b]

[a] Numbers in parentheses are percent inhibitions of PPEP activity-catalyzed hydrolysis of 3.5 μM Ac-C(F)VI[^{3}H]S by 8.5 μM [^{3}H]Ac-C(F)a_1a_2 using Q Sepharose partially purified protein (4.5 μg). [b] PPEP activity detected with Ac-C(F)RL[^{3}H]S was only ~0.5% of that with Ac-C(F)VI[^{3}H]S under the same assay conditions using either Q Sepharose partially purified protein (4.5 μg) or microsomal protein (29 μg).

binder; both the IC_{50} (~0.5 μM) and the K_M (2.2 μM) are lowest among the three tetrapeptides tested.

As shown in Figure 4A, the nonfarnesylated peptide Ac-CVIS does not inhibit PPEP activity even when its concen-

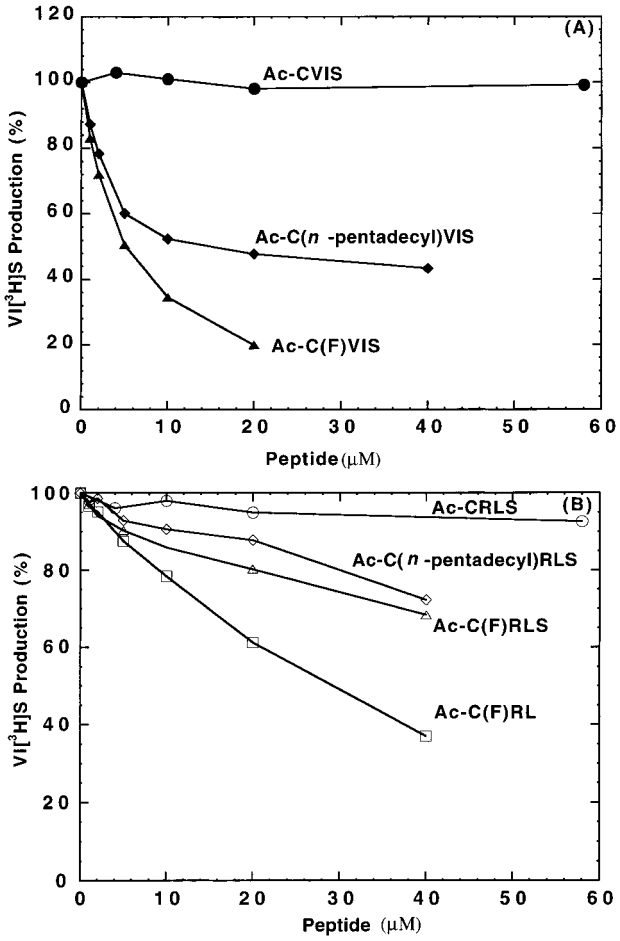

FIGURE 4: (A) Inhibition of PPEP activity-catalyzed hydrolysis of Ac-C(F)VI[^{3}H]S (1 Ci/mmol, 3.5 μM) by nonlipidated and lipidated Ac-CVIS. The assay mixture contained 23 μL of 20 mM Tris-HCl, pH 7, 0.2% CHAPS (0.5 mM of dithiothreitol included in mixture containing Ac-CVIS), 1 μL of various concentrations of peptide inhibitor in DMSO-d_6 or water for Ac-CVIS, 1 μL of substrate stock in DMF, and 25 μL of Q Sepharose partially purified PPEP activity (4.6 μg), added last to initiate the reaction. The reaction was incubated at 37 °C for 40 min. Assays were analyzed by HPLC method i. The percent VI[^{3}H]S produced in the presence of the indicated concentration of the peptide inhibitor as compared to that produced in the absence of this inhibitor is plotted. (B) Inhibition of PPEP activity-catalyzed hydrolysis of Ac-C(F)VI[^{3}H]S (1 Ci/mmol, 3.5 μM) by nonlipidated and lipidated Ac-CRLS. Same conditions as in panel A.

tration is 17 times higher than the concentration of farnesylated substrate. Replacement of the farnesyl group with a straight-chain hydrocarbon (*n*-pentadecyl) leads to a modest reduction in binding to PPEP activity.

We were intrigued by the observation that R is tolerated in the a_1 position as long as a_2 contains a large hydrophobic residue (Table 2), however see below. This was studied in more detail (Figure 4B). Again, the nonfarnesylated peptide, Ac-CRLS does not bind to PPEP activity. Interestingly, Ac-C(F)RL is a better PPEP activity binder than is Ac-C(F)-RLS. This fits with the notion that PPEP activity prefers the most hydrophobic C-terminal extension. Again, replacement of the farnesyl group by the straight-chain hydrocarbon leads to only a modest reduction in PPEP activity binding (compare Ac-C(F)RLS to Ac-C(*n*-pentadecyl)RLS).

Hydrolysis of Radiolabeled Peptides by PPEP Activity. After testing the extensive series of peptides Ac-C(F)a_1a_2 as inhibitors of Ac-C(F)VI[^{3}H]S hydrolysis by partially purified PPEP activity, we prepared a smaller set of radiolabeled tripeptides [^{3}H]Ac-C(F)a_1a_2 and measured the amount of radiolabeled [^{3}H]Ac-C(F) formed in the presence of a fixed amount of partially purified PPEP activity and after a fixed reaction time (90 min). We mainly focused on those peptides that are good binders (Table 2) and a few that are weak binders (IQ, ID, and ST). All reactions give an identical radiolabeled product that can be detected by HPLC. For all test substrates, a minus PPEP activity control was included to ensure that the radiolabeled substrate, which is present in large excess over product, did not interfere with scintillation counting. In those cases where the plus PPEP activity sample yielded <2-fold [^{3}H]Ac-C(F) cpm as compared to the minus enzyme control, the formation of product was confirmed by TLC with fluorography (see Methods). Substrate specificity data are summarized in Table 3 (top). Those peptides of [^{3}H]Ac-C(F)a_1a_2 peptides containing the most hydrophobic a_1a_2 dipeptide (FL, FM, IM, IV, LI, LL, and LM, ~5−10% of peptide substrate hydrolyzed) are hydrolyzed 45−120% as well as [^{3}H]Ac-C(F)VI. The more hydrophilic peptides (a_1a_2 = ID, IQ, or ST) show little, if any, hydrolysis (0−5%). Interestingly, although Ac-C(F)RL binds to PPEP activity (Table 2 and Figure 4B), [^{3}H]Ac-C(F)RL is a poor substrate (6%) for PPEP activity. This result was further confirmed using the [^{3}H]Ac-C(F)a_1a_2 (Table 3, a_1a_2= VI, FL, RL, and ST, numbers in parentheses) as inhibitors (8.5 μM) to compete against Ac-C(F)VI[^{3}H]S substrate (3.5 μM). Again, [^{3}H]Ac-C(F)RL together with [^{3}H]Ac-C(F)VI and [^{3}H]Ac-C(F)FL showed comparable inhibition, whereas [^{3}H]-Ac-C(F)ST showed very little inhibition. These results were consistent with those in Table 2, which shows that the radiolabeled peptides are structurally intact.

On the basis of the above inhibition and substrate specificity results, the next step was to analyze a smaller set of Ac-C(F)a_1a_2X tetrapeptides. We first determined which type of X residue (hydrophilic or hydrophobic) was most preferred by PPEP activity. Ac-C(F)VI[^{3}H]L (1 Ci/mmol) and Ac-C(F)VI[^{3}H]S (1 Ci/mmol) were prepared and tested with Q Sepharaose partially purified PPEP activity. K_M and V_{max} values (Table 3, bottom) for Ac-C(F)VI[^{3}H]L and Ac-C(F)VI[^{3}H]S are very similar. Since V_{max}/K_M for Ac-C(F)-VI[^{3}H]L is 2-fold higher than that for Ac-C(F)VI[^{3}H]S, we decided to use L in the X position of Ac-C(F)a_1a_2X peptides for subsequent studies. Four peptides, Ac-C(F)FM[^{3}H]L, Ac-C(F)LI[^{3}H]L, Ac-C(F)LL[^{3}H]L, and Ac-C(F)LM[^{3}H]L, were prepared and tested with the hope of finding an optimal PPEP activity substrate. Their K_M and V_{max} values are summarized in Table 3, bottom. K_M values for Ac-C(F)LI[^{3}H]L, Ac-C(F)LL[^{3}H]L, and Ac-C(F)LM[^{3}H]L show only modest variation as do the V_{max} values. One purpose for studying the substrate specificity of PPEP activity is to define the best binder(s) and/or substrate(s) for PPEP activity and utilize them for affinity purification of PPEP activity or for the core structure of PPEP activity-specific photolabeling reagents. In this latter context, we synthesized a radiolabeled, benzoylbenzoylated photoprobe based on our best binder, Ac-C(F)LML, by the reaction of the *N*-hydroxysuccinimide ester of 4-benzoyl benzoic acid with CLML followed by enzymatic farnesylation of the peptide. Unfortunately, we failed to specifically photolabel PPEP activity.

The α and β subunits of phosphorylase kinase with C-terminal sequences CAMQ and CLVS, respectively, are farnesylated but not proteolyzed in muscle (*48*). We decided to measure the rate of hydrolysis of ECB-C(F)CLV[^{3}H]S prepared as described previously (*49*). When this peptide and ECB-C(F)CVI[^{3}H]S were added to microsomes at 3 μM each, the phosphorylase kinase-derived peptide was proteolyzed 2-fold slower than the transducin γ-subunit-derived peptide. This result together with the fact that two hydrophobic residues in the a_1a_2 sequence are well tolerated by PPEP activity suggest that phosphorylase kinase β subunit escapes proteolysis in muscle for reasons that are not apparent.

Specificity Studies with Microsomal PPEP Activity. It was of interest to see if the observed trends in relative inhibitor potency with solubilized, partially purified PPEP activity (Table 2) are similar to those with microsomal PPEP activity. A selected set of Ac-C(F)a_1a_2 tripeptides were tested as competitive inhibitors of the hydrolysis of Ac-C(F)VI[^{3}H]S by PPEP activity in rat liver microsomes. In the presence of a fixed concentration of Ac-C(F)VI[^{3}H]S of 3 μM, 40 μM Ac-C(F)VI, Ac-C(F)ID, Ac-C(F)IQ, Ac-C(F)IV, and Ac-C(F)RL inhibited microsomal PPEP activity by 58%, 10% (21% at 100 μM), 18% (35% at 100 μM), 63%, and 35%, respectively. The trend is similar to that seen with solubilized, partially purified PPEP activity, i.e., a_1a_2 dipeptide moieties that are more hydrophilic are less well tolerated by PPEP activity and R at the a_1 position is tolerated to some extent. These results suggest that PPEP activity present in microsomes is the same as that which was solubilized.

Partitioning of Ac-C(F)a_1a_2 Tripeptides into Microsomes. For water-soluble enzymes that operate on water-soluble substrates, the equilibrium dissociation constant for the enzyme−substrate complex, K_s is given by the standard expression K_s = [enzyme][substrate]/[enzyme·substrate]. For an integral membrane enzyme such as PPEP activity, the situation is complicated by the fact that the substrate exists in both the membrane and aqueous phases, while the enzyme remains in the membrane phase. For this situation, the enzyme·substrate equilibrium is described by eq 1 (eq 9 of ref *50*).

$$K_S^{eff} = \frac{(E^*)(S + S^*)}{V_T(E^*\cdot S^*)} = K_S^*[\text{Lipid}]_T\left(1 + \frac{S}{S^*}\right) \qquad (1)$$

Here, the species with an asterisk are in the membrane phase, V_T is the total system volume (aqueous + membrane), and $[\text{Lipid}]_T$ is the total concentration of membrane lipid that S* is accessible to (moles of lipid/V_T). The effective dissociation constant K_S^{eff} and the interfacial dissociation constant K_S^* have been introduced. Note that when the total substrate concentration $[(S + S^*)/V_T]$ is equal to K_S^{eff}, half of PPEP activity will be bound to substrate, and thus K_S^{eff} behaves like a dissociation constant. K_S^* is the mole fraction of substrate in the membrane, X_S^*, that gives E*/E*·S* = 1 (eq 5 of ref *50*).

$$K_S^* = \frac{E^* X_S^*}{E^*\cdot S^*}$$

It is useful to consider two extremes. If most of the substrate is in the membrane phase, eq 2 applies (eq 10 of ref *50*), whereas if most of the substrate is in the aqueous phase, eq 3 applies (eq 11 of ref *50*).

$$K_S^{eff} = K_S^*[\text{Lipid}]_T \qquad (2)$$

$$K_S^{eff} = K_S^*[\text{Lipid}]_T \frac{1}{P_S}\frac{V_w}{V_m} \qquad (3)$$

In eq 3, the equilibrium constant for partitioning of substrate between the membrane and aqueous phases, P_S, is given by the standard equation where V_w is the volume of water phase and V_m is the volume of membrane phase that substrate is accessible to.

$$P_S = \frac{\frac{S^*}{V_m}}{\frac{S}{V_w}}$$

From this analysis, an important point emerges when one considers the substrate specificity of PPEP activity. If two substrates are mostly in the membrane, the difference in observed values of K_S^{eff} (measured by the competitive analysis; see Specificity Studies with Microsomal PPEP Activity) will be equal to the difference in values of their intrinsic affinity for PPEP activity (K_S^* values, eq 2). On the other hand if the two substrates are mainly in the aqueous phase, the difference in observed values of K_S^{eff} will reflect a combination of differences in K_S^* and in P_S. Thus, for example, Ac-C(F)ID may be a poorer inhibitor of PPEP activity than Ac-C(F)VI not because it has intrinsically lower affinity for the active site of the enzyme but because it is partitioned less well into membranes.

To explore this issue, the same set of Ac-C(F)a_1a_2 tripeptides that were used in substrate specificity studies with microsomal PPEP activity were submitted to membrane partitioning studies. These peptides were mixed with microsomal membranes under the identical condition used for kinetic studies, and the fractions of peptide in the membrane and the supernatant were measured after pelleting the membranes in an ultracentrifuge. Controls were also carried out in which membranes were omitted. HPLC was performed on all samples, and the amount of peptide in the supernatant and membranes is expressed as a percentage of the amount of peptide detected in control experiments. The fractions bound to the membranes and in the supernatant are, respectively, 34% and 61% for Ac-C(F)VI, 12% and 86% for Ac-C(F)ID, 24% and 73% for Ac-C(F)IQ, 35% and 63% for Ac-C(F)IV, and 79% and 21% for Ac-C(F)RL. It can now be stated that the peptide Ac-C(F)IQ is a poorer binder to PPEP activity as compared to more hydrophobic peptides not because of differences in membrane partitioning but because of a lower intrinsic affinity for PPEP activity. Ac-C(F)ID is a very poor PPEP activity binder because of a combination of decreased binding to membranes and of lower intrinsic affinity for PPEP activity. Since Ac-C(F)RL partitions into membranes more favorably than the other peptides, its intrinsic affinity for PPEP activity is somewhat overestimated by the inhibition data described under Specificity Studies with Microsomal PPEP Activity. The enhanced binding of the arginine-containing peptide and the

poorer binding of the aspartate-containing peptide to membranes is consistent with the fact that biological membranes have a net negative charge due to the presence of acidic phospholipids. These issues of substrate specificity, which are often overlooked, must be considered for any integral or peripheral membrane protein and, in fact, for any water-soluble enzyme that is analyzed in the presence of membranes.

ACKNOWLEDGMENT

We thank our colleagues Drs. Li Liu, Paul Mcgeady, and Kohei Yokoyama for helpful discussions.

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BI972289B