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Evaluation of substrate and inhibitor binding to yeast and human isoprenylcysteine carboxyl methyltransferases (Icmts) using biotinylated benzophenone-containing photoaffinity probes

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ABSTRACT

Isoprenylcysteine carboxyl methyltransferases (Icmts) are a class of integral membrane protein methyltransferases localized to the endoplasmic reticulum (ER) membrane in eukaryotes. The Icmts from human (hIcmt) and *Saccharomyces cerevisiae* (Ste14p) catalyze the α-carboxyl methyl esterification step in the post-translational processing of CaaX proteins, including the yeast **a**-factor mating pheromones and both human and yeast Ras proteins. Herein, we evaluated synthetic analogs of two well-characterized Icmt substrates, *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) and the yeast **a**-factor peptide mating pheromone, that contain photoactive benzophenone moieties in either the lipid or peptide portion of the molecule. The AFC based-compounds were substrates for both hIcmt and Ste14p, whereas the **a**-factor analogs were only substrates for Ste14p. However, the **a**-factor analogs were found to be micromolar inhibitors of hIcmt. Together, these data suggest that the Icmt substrate binding site is dependent upon features in both the isoprenyl moiety and upstream amino acid composition. Furthermore, these data suggest that hIcmt and Ste14p have overlapping, yet distinct, substrate specificities. Photocrosslinking and neutravidin–agarose capture experiments with these analogs revealed that both hIcmt and Ste14p were specifically photolabeled to varying degrees with all of the compounds tested. Our data suggest that these analogs will be useful for the future identification of the Icmt substrate binding sites.

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1. Introduction

Many eukaryotic proteins are initially synthesized with a C-terminal amino acid CaaX motif that signals a series of post-translational modifications including isoprenylation of the cysteine (C) by either a farnesyl or geranylgeranyl moiety, proteolysis of the –aaX residues and α-carboxyl methyl esterification of the newly exposed cysteine residue [1–3]. CaaX proteins include the Ras superfamily of small GTPases [4,5], Rheb, the nuclear lamins, the Rho family of GTPases and the γ subunits of heterotrimeric G proteins [2,6–8].

The only enzymes known to methyl esterify the α-carboxylate group of CaaX proteins are the isoprenylcysteine carboxyl methyltransferases (Icmts), a family of integral membrane proteins localized to the endoplasmic reticulum (ER) [2,6,9–12]. Ste14p from *Saccharomyces cerevisiae*, the founding member of the Icmt family of enzymes, is a 26-kDa integral membrane protein with six putative transmembrane helices [13,14]. The human enzyme, hIcmt, which shares 41% identity and 63% similarity with Ste14p, is a 33-kDa membrane protein with eight putative transmembrane helices [15–17]. Interestingly, hIcmt functionally complements the mating defect of a Δ*ste14* strain by methylating the **a**-factor peptide, suggesting that the enzymes have overlapping substrate specificities [15]. In addition to CaaX proteins and peptides, numerous small molecules such as *N*-acetyl-*S*-farnesyl-L-cysteine (AFC), *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC), and farnesyl thiopropionic acid (FTP) have been shown to be substrates for both human and yeast Icmts [18,19], while other compounds have shown specificity for the yeast enzyme [17].

Aside from these few examples, little is known about the differences in substrate specificity between the yeast and human enzymes, nor is it known how and where the substrates bind to these Icmts. A recent 3.4 Å crystal structure of the prokaryotic Icmt ortholog *Ma*-ICMT has been published that revealed important

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well-conserved structural features of the binding pocket for the co-substrate S-adenosyl-L-methionine (SAM) [20]. The structure also showed a conserved access tunnel for lipidated substrates that is comprised of residues both in the C-terminal SAM-binding domain and those in the N-terminal half of the protein [20]. The N-terminal segment of the protein is thought to confer substrate specificity for lipid substrates. However, poor sequence conservation between *Ma*-ICMT and the eukaryotic Icmts in the N-terminal half precluded a definitive analysis of residues important for binding the isoprene moieties and thus, different approaches must be taken to identify these key amino acids.

Benzophenone-modified substrate analogs have been used previously to interrogate the protein binding sites for farnesyl and geranylgeranyl moieties. The utility of this approach was first demonstrated in experiments designed to probe the interaction between the CaaX protein Rho and its regulator RhoGDI [21]. In that study, an isoprenoid-containing cysteine analog bearing the benzophenone label in the lipid itself was used to demonstrate that the isoprene group itself specifically interacts with RhoGDI. Most recently, a series of benzophenone-modified peptide analogs based on the yeast **a**-factor sequence was developed and used to examine the activity of yeast CaaX protease Ras converting enzyme 1 (Rce1p). These experiments demonstrated that yeast Rce1p recognized the modified analogs as substrates and each analog specifically photoaffinity labeled the protein [22,23]. Similarly, carboxyl methylated, photoactive analogs of the **a**-factor mating pheromone peptide were shown to functionally interact with the **a**-factor receptor, Ste3p [24].

In this study, we synthesized and examined several analogs of AFC and farnesylated **a**-factor peptides that contained both a biotin tag and a photoactive benzophenone moiety, either in the isoprene unit or in the peptide region, for their ability to bind and act as substrates or inhibitors of hIcmt and Ste14p. The benzophenone group allowed for specific photoaffinity labeling of the substrate binding site and the biotin moiety allowed for isolation of the labeled protein from a crude membrane mixture. Using kinetic studies, we determined that the AFC analogs were substrates for both enzymes. The **a**-factor analogs were only substrates for Ste14p but were competitive inhibitors of hIcmt. Furthermore, using photocrosslinking experiments followed by isolation with neutravidin–agarose beads, we determined that both Ste14p and hIcmt were labeled by all of the analogs tested, albeit to varying degrees, under saturating conditions.

2. Materials and methods

2.1. Chemistry

2.1.1. Materials

All solvents and reagents used for the solid-phase peptide synthesis of the photoactivatable peptides were of analytical grade and purchased from Peptides International (Louisville, KY), NovaBioChem® (Nohenbrunn, Germany), or Sigma–Aldrich (St. Louis, MO). *N*-Acetyl-*S*-farnesyl-L-cysteine (AFC) was synthesized in the Gibbs laboratory (Purdue University) as previously described [25]. High performance liquid chromatography grade acetonitrile (CH_3CN), dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), and H_2O were purchased from Fisher Scientific (Springfield, NJ), OmniSolv® (Charlotte, NC), or Sigma–Aldrich. NHS-PEG$_4$-Biotin was obtained from Thermo Scientific (Waltham, MA).

2.1.2. Synthesis and chemical characterization of photoaffinity analogs

Detailed descriptions for the synthesis and chemical characterization of the photoaffinity analogs Am-bpBFC BPA Analog (**2**), F-bpBFC AFC Analog (**3**), Biotin-Peg$_4$-YIIKGVFWDPAC (**4**), Biotin-Peg$_4$-YIIKGVFWDPAC(C$_5$-meta-Bp) (**7a**), Biotin-Peg$_4$-YIIKGVFWDPAC(C$_5$-para-Bp) (**7b**), Biotin-Peg$_4$-YIIKGVFWDPAC(C$_{10}$-meta-Bp) (**8a**) and Biotin-Peg$_4$-YIIKGVFWDPAC(C$_{10}$-para-Bp) (**8b**) are found in Supplementary data.

2.2. Biochemical evaluation

2.2.1. Materials

S-Adenosyl-L-[^{14}C-methyl] methionine ([^{14}C]-SAM) was purchased from Perkin Elmer (Waltham, MA). α-*myc* monoclonal antibody, goat α-mouse IgG and goat α-rabbit IgG were purchased from Invitrogen (Carlsbad, CA). The α-Ste14 polyclonal antibody was a gift from Dr. S. Michaelis (The Johns Hopkins University School of Medicine). The neutravidin coated agarose beads and SuperSignal West Pico enhanced chemiluminescence (ECL) were purchased from Pierce (Rockford, IL).

2.2.2. Yeast strains and crude membrane preparations from yeast cells

His$_{10}$myc$_3$N-Ste14p (His-Ste14p) and His$_{10}$myc$_3$N-hIcmt (His-hIcmt) yeast strains were cloned and expressed as previously described [9,17]. Crude membranes were prepared as described previously with minor modifications [9]. After centrifugation at 100,000*g*, the membrane pellet was resuspended in 10 mM Tris–HCl, pH 7.5, aliquoted, flash frozen in liquid N_2 and stored at −80 °C.

2.2.3. In vitro methyltransferase vapor diffusion assay

Reactions were performed as described previously [26]. All inhibition studies were completed as detailed earlier [17]. Crude membrane preparations derived from a *Δste14* strain transformed with an empty vector were used as the negative control for these experiments.

2.2.4. Photocrosslinking and neutravidin–agarose pull-down assays

Photocrosslinking assays were performed as described previously, with minor modifications [22]. Briefly, 100 μg of crude membrane preparation expressing either His-Ste14p or His-hIcmt in 100 mM Tris–HCl, pH 7.5 were incubated in the presence of saturating concentrations of the photoaffinity analogs and incubated at 4 °C for 10 min. After incubation, the samples were irradiated with UV light (365 nm) in 96-well plates for 40 min on ice. Following photocrosslinking, unreacted analog was removed by chloroform/methanol extraction [27]. The resulting protein samples were solubilized in 400 μL of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate)/10% SDS and incubated with 50 μL of a 50% neutravidin/RIPA bead slurry for 2 h at room temperature. Following incubation, the beads were centrifuged at 13,000*g* for 1 min and washed three times with RIPA/10% SDS. The crosslinked protein was eluted from the neutravidin beads by the addition of 50 μL of 2× SDS sample buffer (0.5 M Tris–HCl, pH 6.8, 30% sucrose (w/v), 10% sodium dodecylsulfate (w/v), 3.5 M 2-mercaptoethanol and 0.1% bromophenol blue (w/v)). The His-Ste14p samples were heated for 30 min at 65 °C and the His-hIcmt samples were incubated at room temperature overnight. The proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose (0.22 μm). The nitrocellulose membrane was blocked at room temperature for 2 h in 20% (w/v) non-fat dry milk in phosphate-buffered saline with Tween-20 (137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 0.05% (v/v) Tween-20, pH 7.4) (PBST). The blocked membrane was then incubated for 2 h at room temperature with primary antibody (1:1000 α-Ste14p) or (1:10,000 α-myc) in 5% (w/v) non-fat dry milk in PBST for His-Ste14p or His-hIcmt, respectively. The membrane was washed in PBST three times and then incubated

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