# Cross-Linking of a DOPA-Containing Peptide Ligand into Its G Protein-Coupled Receptor†

George K. E. Umanah,‡ Cagdas Son,‡,§ FaXiang Ding,|| Fred Naider,||,⊥,# and Jeffrey M. Becker*,‡

*Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996, and Department of Chemistry and Macromolecular Assemblies Institute, College of Staten Island, and Graduate School and University Center, City University of New York, New York, New York 10314*

*Received November 6, 2008; Revised Manuscript Received January 16, 2009*

ABSTRACT: The interaction between a 3,4-dihydroxyphenylalanine (DOPA) labeled analogue of the tridecapeptide α-factor (W-H-W-L-Q-L-K-P-G-Q-P-M-Y) and Ste2p, a *Saccharomyces cerevisiae* model G protein-coupled receptor (GPCR), has been analyzed by periodate-mediated cross-linking. Chemically synthesized α-factor with DOPA substituting for tyrosine at position 13 and biotin tagged onto lysine$^7$([Lys$^7$(BioACA),Nle$^{12}$,DOPA$^{13}$]α-factor; Bio-DOPA-α-factor) was used for cross-linking into Ste2p. The biological activity of Bio-DOPA-α-factor was about one-third that of native α-factor as determined by growth arrest assay and exhibited about a 10-fold lower binding affinity to Ste2p. Bio-DOPA-α-factor cross-linked into Ste2p as demonstrated by Western blot analysis using a neutravidin-HRP conjugate to detect Bio-DOPA-α-factor. Cross-linking was inhibited by excess native α-factor and an α-factor antagonist. The Ste2p−ligand complex was purified using a metal ion affinity column, and after cyanogen bromide treatment, avidin affinity purification was used to capture Bio-DOPA-α-factor-Ste2p cross-linked peptides. MALDI-TOF spectrometric analyses of the cross-linked fragments showed that Bio-DOPA-α-factor reacted with the Phe$^{55}$−Met$^{69}$ region of Ste2p. Cross-linking of Bio-DOPA-α-factor was reduced by 80% using a cysteine-less Ste2p (Cys59Ser). These results suggest an interaction between position 13 of α-factor and residue Cys$^{59}$ of Ste2p. This study is the first to report DOPA cross-linking of a peptide hormone to a GPCR and the first to identify a residue-to-residue cross-link between Ste2p and α-factor, thereby defining a specific contact point between the bound ligand and its receptor.

G protein-coupled receptors (GPCRs)[1] are a large family of integral membrane proteins associated with signaling systems present in mammalians, plants, protozoans, fungi, and metazoans (*1*). Malfunctions of GPCRs contribute to diseases such as Alzheimer's, Parkinson's, diabetes, color blindness, asthma, depression, hypertension, stress, cardiovascular, and immune disorders (*2, 3*). All GPCRs share a common structure consisting of seven transmembrane domains (TMDs) connected by three extracellular and three intracellular loops (*1, 4, 5*). Upon binding of their ligands, GPCRs undergo conformational changes that transduce the signal into the cell by activating a cascade of protein−protein interactions initiated through a heterotrimeric G protein complex (*6*).

Ste2p, the *Saccharomyces cerevisiae* α-factor pheromone receptor, has been studied as a model for peptide-responsive GPCRs (*7, 8*). Though Ste2p does not share recognizable sequence similarity with mammalian GPCRs or even with Ste3p, the **a**-factor pheromone receptor of yeast, all GPCRs have strong structural and functional similarities (*9, 10*). For example, the packing and interactions between the fifth and sixth transmembrane domains are essential for proper signal transduction to the G protein in both rhodopsin and Ste2p (*11, 12*). Though there have been considerable studies on the GPCR binding sites of small ligands, less is known about the binding sites of peptide-responsive GPCRs.

Studies on the tridecapeptide α-factor pheromone (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) have contributed to the understanding of structure and function of peptide hormones. Alanine scanning studies of α-factor indicated that Trp$^1$−Leu$^4$ residues are associated with receptor activation and signal transduction; Lys$^7$−Gln$^{10}$ residues are involved in formation of the biologically active conformation, and Gln$^{10}$−Tyr$^{13}$ residues are essential for receptor

† This work was supported by Grants GM-22086 and GM-22087 from the National Institute of General Medical Sciences of the National Institutes of Health.

* Corresponding author. Tel: 865-974-3006. Fax: 865-974-4007. E-mail: jbecker@utk.edu.

‡ University of Tennessee.

§ Current address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

|| Department of Chemistry and Macromolecular Assemblies Institute, College of Staten Island, CUNY.

⊥ Graduate School and University Center, CUNY.

# F.N. is currently The Leonard and Esther Kurtz Term Professor at the College of Staten Island.

[1] Abbreviations: BioACA, biotinylaminocaproic acid; Bio-DOPA-α-factor, an analogue of α-factor with DOPA replacing Tyr$^{13}$ and biotin attached to Lys$^7$ ([K$^7$(BioACA),Nle$^{12}$,DOPA$^{13}$]α-factor); DMF, dimethylformamide; DOPA, 3,4-dihydroxyphenylalanine; DTT, 1,4-dithiothreitol; Fmoc, fluorenyloxycarbonyl; GPCR, G protein-coupled receptor; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MLT, medium lacking tryptophan; Nle, norleucine; tBu, *tert*-butyl; PNA, peptide nucleic acid; SDS, sodium dodecyl sulfate; Ste2p, α-factor receptor encoded by the *STE2* gene; TFA, trifluoroacetic acid; TMD, transmembrane domain. Standard one-letter abbreviations for amino acids are used.

10.1021/bi802061z CCC: $40.75 
Published on Web 01/16/2009

binding (*13*). Photoreactive cross-linking studies, using 4-benzoyl-L-phenylalanine (Bpa) containing α-factor analogues at different positions, indicated that positions 1, 3, and 13 in α-factor interacted with residues on the extracellular face of Ste2p (*14*−*16*). The N-terminus of α-factor was shown to interact with Ste2p residues from extracellular ends of the fifth, sixth, and seventh TMDs and parts of the second and third extracellular loops, whereas the C-terminus interacted with part of the first transmembrane domain (*14, 16, 17*). All of these studies revealed that generation of covalently linked ligand−receptor conjugates followed by detailed product analysis was an attractive approach to identify and map residues involved in Ste2p−ligand interactions. Nevertheless, limitations of using the cross-linking approaches performed to date are low yield of product and multiple cross-linked products making it difficult to define the exact residue−residue interactions involved in GPCR binding.

Recently, compounds containing 3,4-dihydroxyphenylalanine (DOPA) have been shown to cross-link efficiently to proteins (*18*). DOPA-containing compounds were oxidized by sodium periodate to form an *o*-quinone intermediate that could be attacked by a nearby nucleophile resulting in a stable, covalent cross-link. The *o*-quinone intermediate was shown to act as an electrophile that formed adducts with cysteine or histidine through the Michael addition reaction and also with lysine through the Schiff base formation (*18, 19*). Unlike most chemical cross-linking approaches, DOPA cross-linking occurs in high yield with little or no nonspecific products observed even in complex protein mixtures (*20*). This method has been utilized to identify the yeast Rpt6/Sug1 and Rpt4/Sug2 proteins as the direct targets of Gal4 transcriptional activation domains within the 26S proteasome (*21*). DOPA contained in RIP-1 (*r*egulatory particle *I*nhibitor *p*eptoid-1) was also used to identify the yeast RIP-1 receptor (*22*).

Previously, we reported that an α-factor analogue containing Bpa at position 13 cross-linked into a portion of Ste2p comprising residues $Phe^{55}$−$Arg^{58}$ (*14*). However, the exact residue of Ste2p that interacted with position 13 of α-factor was not defined. In this study we investigated the periodate-mediated chemical cross-linking of [$Lys^7$(BioACA),$Nle^{12}$,$DOPA^{13}$]α-factor (Bio-DOPA-α-factor) into Ste2p. This α-factor analogue was a potent agonist and bound with 100 nM affinity to Ste2p. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) analysis revealed that Bio-DOPA-α-factor cross-linked to a fragment of Ste2p comprising residues $Phe^{55}$−$Met^{69}$. Cross-linking of the DOPA α-factor analogue into a Ste2p mutant (Cys59Ser) devoid of cysteine was greatly diminished, suggesting that $DOPA^{13}$ of the α-factor analogue interacted directly with $Cys^{59}$ of Ste2p. This represents the first time that DOPA oxidative cross-linking has been used to link a peptide hormone to its GPCR receptor and demonstrates the general utility of this method for studying contact points of biologically active peptides with their cognate receptors.

## EXPERIMENTAL PROCEDURES

*Media, Reagents, Strains, and Plasmids. S. cerevisiae* strain LM102 [*MAT***a**, *bar1, leu2, ura3, FUS1-lacZ::URA3, ste2*Δ (*23*)] was used for growth arrest and binding assays, and the protease-deficient strain BJS21 [*MAT***a**, *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::Kan*$^R$ (*24*)] was used for protein isolation and immunoblot analysis. The plasmid pBEC1 containing C-terminal FLAG and His-tagged *STE2* (*23*) was transformed by the method of Geitz (*25*) into LM102 and BJS21 cells. Transformants were selected by growth on yeast media (*26*) lacking tryptophan (designated as MLT) to maintain selection for the plasmid. The cells were cultured in MLT and grown to midlog phase at 30 °C with shaking (200 rpm) for all assays.

*Synthesis and Characterization of Peptides.* The tridecapeptide pheromone α-factor (WHWLQLKPGQPN$le^{12}$-$DOPA^{13}$) and [$K^7$(biotinylaminocaproate),$DOPA^{13}$]α-factor (designated Bio-DOPA-α-factor) were synthesized using automated solid-phase peptide synthesis with Fmoc/OtBu protection schemes. The hydroxyl groups of DOPA were protected as the acetonide derivative. All protected amino reagents were purchased from Advanced Chem Tech (Louisville, KY), and all solvents and reagents were of the highest purity available. α-Factor and α-factor analogues used in this study all contain norleucine in place of the native $Met^{12}$ residue. Norleucine is isosteric with methionine, and [$Nle^{12}$]α-factor is isoactive and has the same receptor affinity as α-factor (*27*). The syntheses of [$Nle^{12}$]α-factor, $desTrp^1$, $desHis^2$[$Nle^{12}$]α-factor, and [$biotin^7$]α-factor were done as described previously (*14, 28*).

*Synthesis of Fmoc-WHWLQLKPGQPNleDOPA-OH.* To preswollen Wang resin (0.145 g, 0.1 mmol, 0.69 mmol/g) in DMF (10 mL) was added Fmoc-DOPA(acetonide)-OH (115 mg, 0.25 mmol), *N*-hydroxybenzotriazole (34 mg, 0.25 mmol), 4-dimethylaminopyridine (3.05 mg, 0.025 mmol), and diisopropylcarbodiimide (31.55 mg, 0.25 mmol), and the resulting mixture was stirred at room temperature for 1 h. The mixture was filtered, and the resin was treated again with the reagents above in DMF (10 mL) at room temperature for 1 h. After filtration and washing with DMF (10 mL × 2), the resin was further treated with acetic anhydride (102 mg, 1.0 mmol) and *N*,*N*-diisopropylethylamine (128 mg, 1.0 mmol) in DMF (10 mL) for 30 min to cap unreacted hydroxyl groups, followed by washing with DMF (3 × 10 mL). The Fmoc-DOPA(acetonide)-O-Wang resin (0.1 mmol) was loaded on a Model 433A solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA) using 0.1 mmol of single coupling chemistry and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole as coupling reagents. After completing the chain assembly, the resin was dried under vacuum. To cleave the peptide, the dry resin (550 mg) obtained above was added to a mixture of phenol (0.75 g), ethanedithiol (0.5 mL), thioanisole (0.5 mL), water (0.5 mL), and 10 mL trifluoroacetic acid, and the resulting mixture was stirred for 1.5 h at room temperature. After filtration the volume of the filtrate was reduced by rotary evaporation *in vacuo*, the resulting residue was treated with 100 mL of ethyl ether, and the precipitate was collected by filtration. The crude peptide (180 mg) was purified on a preparative HPLC C18 column (19 mm × 300 mm) using gradient from 30% to 70% acetonitrile/$H_2O$ (0.1% TFA) over 90 min to give pure Fmoc-WHWLQLKPGQPNleDOPA-OH (70 mg).

*Synthesis of WHWLQLKPGQPNleDopa-OH.* To a vial covered with aluminum foil containing Fmoc-WHWLQLKPGQPNleDOPA-OH (12 mg) and DMF (1 mL) was added

piperidine (0.1 mL, 18% in DMF) at 4 °C under an argon atmosphere, and the solution was stirred at 4 °C for 30 min. The solution was neutralized by 2% HCl; after filtration, the filtrate was loaded onto a preparative HPLC (C18 column, 19 mm × 300 mm) using a gradient of 20−50% acetonitrile (0.1% TFA) in water (0.1% TFA). Seven milligrams of pure WHWLQLKPGQPNleDOPA-OH ([DOPA$^{13}$]α-factor) was obtained. Yield: 64%. MW: calcd, 1681.97; found, 1680.9.

*Synthesis of WHWLQLK(BiotinACA)PGQPNleDOPA-OH.* To the solution of Fmoc-WHWLQLKPGQPNleDopa-OH (22 mg, 5.16 μmol) in DMF (1 mL) and sodium borate (1 mL, 50 mM) was added biotinamidohexanoic acid *N*-hydroxysuccinimide ester (7.04 mg, 15.48 μmol) at 4 °C. The solution was stirred for 1 h (when HPLC indicated that the starting material had been converted to product), neutralized by 2% HCl, and filtered, and the filtrate was loaded on preparative HPLC (C18 column, 19 × 300 mm) using a gradient from 20% to 70% acetonitrile (0.1% TFA)/water (0.1% TFA). Highly purified Fmoc-WHWLQLK(BioACA)-PGQPNleDOPA-OH (16 mg; 72% yield) was recovered. This Fmoc-peptide (16 mg) was dissolved in DMF (1.2 mL) at 4 °C, and piperidine (66 μL, 18% in DMF) was added. The solution was stirred for 1 h and then was neutralized and filtered, and the filtrate was loaded on preparative HPLC using a gradient of 20−50% acetonitrile (0.1% TFA)/water (0.1% TFA). WHWLQLK(BioACA)PGQPNleDOPA-OH, 5.4 mg, was obtained. Yield: 34%. MW: calcd, 2021.43; obsd, 2022.56 (Figure 1B).

*MALDI-TOF Analysis.* For matrix-assisted laser desorption ionization (MALDI) analysis the peptides were resuspended in 50:50 water−acetonitrile with 0.1% trifluoroacetic acid (TFA) at a final concentration of 0.1 μg/μL. A 20 mg/mL α-cyano-4-hydroxy-*trans*-cinnamic acid (α-ACHA; Sigma/Aldrich Chemical Co., St. Louis, MO) matrix was prepared by dissolving recrystallized α-ACHA in 50:50 water−acetonitrile with 0.1% TFA. An equal volume (0.5 μL) of peptide solution was mixed with matrix before spotting on the MALDI plate. The MADLI-TOF spectra were acquired on a Bruker Daltonics (Boston, MA) Microflex using the reflector method. The tandem mass spectrometry (MS/MS) data were acquired by MALDI postsource decay on a Bruker Daltonics Microflex. The interpretation of the MS/MS data was carried out using Bruker Daltonics BioTools software.

*Growth Arrest Assays.* LM102 cells expressing C-terminal FLAG and His-tagged Ste2p were grown at 30 °C in MLT, harvested, washed three times with water, and resuspended at a final concentration of 5 × 10$^6$ cells/mL (*24*). Cells (1 mL) were combined with 3.5 mL of agar noble (1.1%) and poured as a top agar lawn onto a MLT medium agar plate. Filter disks (BD, Franklin Lakes, NJ) impregnated with α-factor or various α-factor analogues were placed on the top agar. The plates were incubated at 30 °C for 18 h and then observed for clear halos around the disks. The experiment was repeated at least three times, and reported values represent the mean of these tests.

*Binding Competition Assays.* This assay was performed using LM102 cells expressing C-terminal FLAG and His-tagged Ste2p. Tritiated [$^3$H]-α-factor (10.2 Ci/mmol, 12 μM) prepared as previously described (*24, 27*) was used in competition binding assays on whole cells. The cells were grown at 30 °C in MLT, harvested, washed three times with YM1 [0.5 M potassium phosphate (pH 6.24) containing 10 mM TAME, 10 mM sodium azide, 10 mM potassium fluoride, and 1% BSA], and adjusted to a final concentration of 2 × 10$^7$ cells/mL in YM1 plus protease inhibitors (YM1i (*29*)). For the competition binding studies, cells (600 μL) were combined with 150 μL of ice-cold 5× YM1i supplemented with 6 nM [$^3$H]-α-factor in the presence or absence α-factor or α-factor analogues and incubated at room temperature for 30 min. The final concentrations of α-factor and α-factor analogues ranged from 0.5 × 10$^{-10}$ to 1 × 10$^{-6}$ M. After incubation, triplicate samples of 200 μL aliquots were filtered and washed over glass fiber filter mats using the standard cell harvester (Skatron Instruments, Sterling, VA) and placed in scintillation vials. The radioactivity ($^3$H) on the filter was counted by liquid scintillation spectroscopy. The binding data were analyzed by nonlinear regression analysis for one-site competition binding using Prism software (GraphPad Software, San Diego, CA) to determine the binding affinity ($K_d$) and potency ($EC_{50}$) for each peptide. The $K_i$ values were calculated by using the equation of Cheng and Prusoff, where $K_i = EC_{50}/(1 + [\text{ligand}]/K_d)$ (*17*).

*Chemical Cross-Linking.* BJS21 cells expressing C-terminal FLAG and His-tagged *STE2* were grown and total cell membranes isolated as previously described (*24*). Protein concentration was determined by Bio-Rad (Bio-Rad, Hercules, CA) protein assay (*23*). The membranes were resuspended in NE buffer (20 mM HEPES, 20% glycerol, 100 mM KCl, 12.5 mM EDTA, 0.5 mM DTT (*18*)) incubated with Bio-DOPA-α-factor (1 μM) in the presence or absence of 100 μM α-factor (WHWLQLKPGQPNle$^{12}$Y), 100 μM α-factor antagonist (desW$^1$desH$^2$WLQLKPGQPNle$^{12}$Y) (*28*), 100 μM α-factor synergist (WHWLQLKPGQP) (*28*), or 2 μg of BSA (bovine serum albumin) for 30 min at room temperature. For periodate mediated cross-linking, a final concentration of 1.0 mM $NaIO_4$ was added to the mixture and incubated for 2 min. A final concentration of 100 mM DTT (1,4-dithiothreitol) was used to quench the reaction (*18*). The cross-linked membranes were washed three times with CAPS buffer (*N*-cyclohexyl-3-aminopropanesulfonic acid (Sigma, St. Louis, MO), 10 mM, pH 10) by centrifugation to remove nonbound Bio-DOPA. The washed, cross-linked samples were fractionated by SDS−PAGE and then immunoblotted. The blots were probed with an antibody directed against the N-terminal 60 amino acids of Ste2p (generously provided by J. Konopka (*30*)) and with neutravidin-HRP conjugate (Pierce, Rockford, IL) to detect the biotin tag on Bio-DOPA pheromone covalently linked to the Ste2p. The signals generated were analyzed using Quantity One software (version 4.5.1) on a Chemi-Doc XRS photodocumentation system (Bio-Rad, Hercules, CA).

*Purification of Intact Cross-Linked Ste2p.* The cross-linked Ste2p was enriched using His-Select HC nickel affinity gel (Sigma/Aldrich Chemical Co., St. Louis, MO) following the manufacturer's directions. Approximately 10 mg of cell membrane containing cross-linked Ste2p were resuspended in ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) with protease inhibitors (PMSF, pepstatin A, and leupeptin) and incubated overnight at 4 °C with end-over-end mixing and then centrifuged at 15000*g* for 30 min to remove nonsoluble material. The solubilized proteins were then mixed with His-HC-nickel gel and incubated at 4 °C with end-over-end mixing for 1 h. The gel was collected by centrifugation at low speed (500*g*,

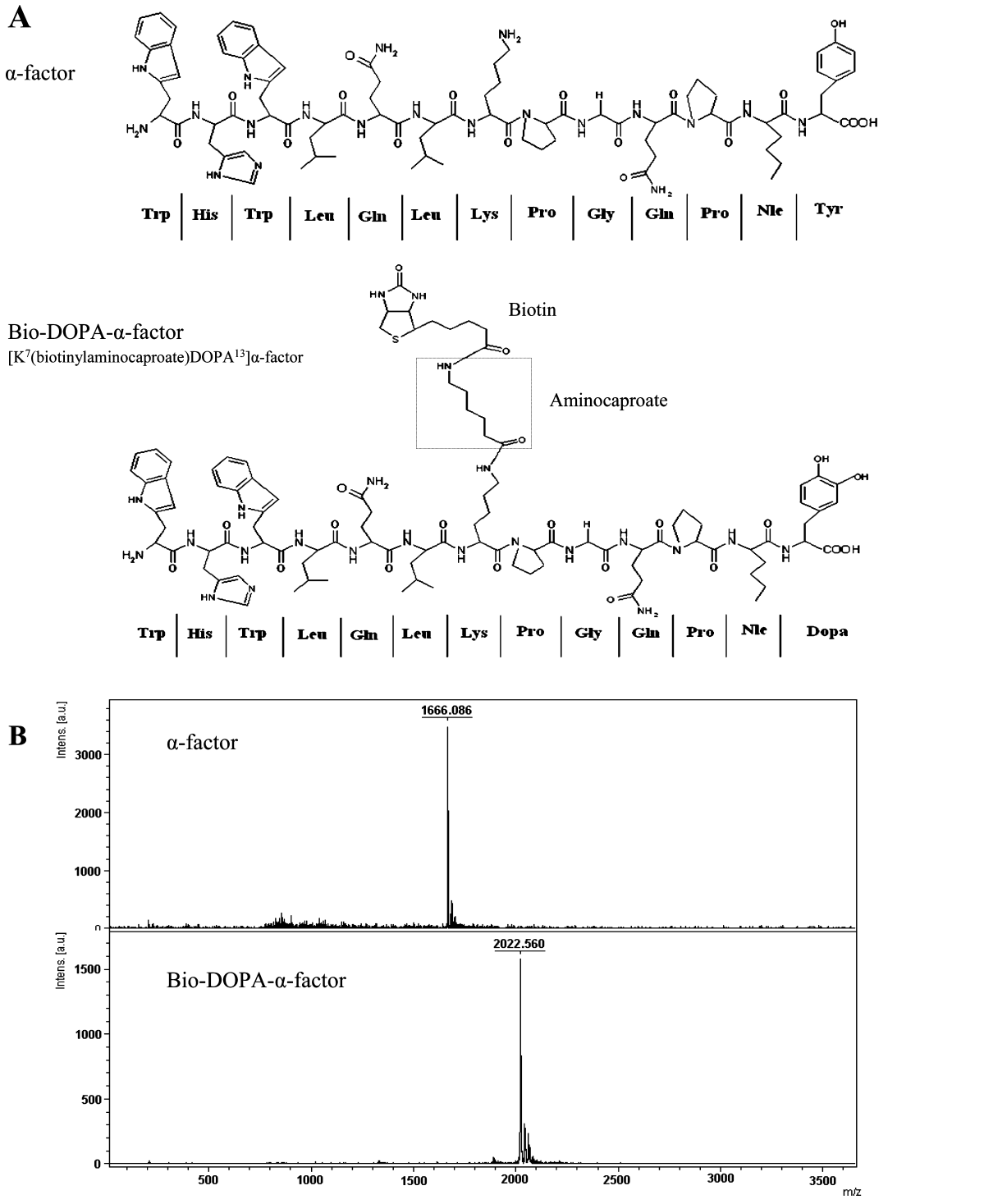


FIGURE 1: Structures and mass spectrometric analysis of α-factor and [$K^7$(biotinylaminocaproate),3,4-dihydroxyphenylalanine(DOPA)$^{13}$]α-factor, abbreviated as Bio-DOPA-α-factor. (A) The $Tyr^{13}$ residue of α-factor is replaced by DOPA, and biotin is conjugated through its carboxyl group to the ϵ-amine of $Lys^7$ using aminocaproate as a linker. (B) The observed mass of the peptides as determined by MALDI-TOF were similar to masses predicted by the PROWL peptide mass prediction tools (*32*): α-factor, 1665.96 Da; Bio-DOPA-α-factor, 2021.43 Da.

1 min) and resuspended and collected four times in wash buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, and 5 mM imidazole). Ste2p was eluted by resuspending the resin in 1 mL of ice-cold elution buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, and 250 mM imidazole) and incubated at 4 °C with end-over-end mixing for 10 min. The resin was pelleted by centrifugation (2000*g*, 1 min) and the supernatant, containing the eluted Ste2p, transferred to a fresh tube. Purity and concentration of samples were estimated by Coomassie blue and silver staining of SDS−PAGE gels (data not shown). The samples were also analyzed by immunoblotting using an antibody directed against Ste2p (*30*) and with neutravidin-HRP conjugate to detect the biotin tag on Bio-DOPA covalently linked to the Ste2p.

*Digestion of Cross-Linked Ste2p.* The enriched cross-linked Ste2p samples eluted from the His-nickel column were digested with cyanogen bromide (CNBr). The eluted samples

containing Ste2p (~20 μg) were dried by vacuum centrifugation (Thermo Scientific, Waltham, MA) and then dissolved in 100% trifluoroacetic acid (TFA) containing 10 mg/mL CNBr. Deionized distilled water ($ddH_2O$) was then added to adjust the final TFA concentration to 80%, and the samples were incubated at 37 °C in the dark for 18 h (*24, 31*). The samples were dried by vacuum centrifugation and washed three times with $ddH_2O$, and then 1 M Tris (pH 8.0) was added to neutralize the acidic mixture.

*Purification of Cross-Linked Ste2p Fragments.* Fragments from the CNBr digestion of cross-linked Ste2p were resuspended in PBS buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7), mixed with monomeric avidin resin (Pierce Thermo Scientific, Rockford, IL), and incubated for 6 h at 4 °C with end-over-end mixing (*16*). The resin was collected by centrifugation at low speed (1000*g*, 1 min) and resuspended and collected four times in PBS buffer. The cross-linked Ste2p fragments were eluted by resuspending the resin in 200 μL of ice-cold elution buffer (0.1 M glycine, pH 2.5) and incubating at 4 °C with end-over-end mixing for 5 min. The resin was pelleted by centrifugation (2000*g*, 1 min) and the supernatant, containing the eluted cross-linked Ste2p fragments, transferred to a fresh tube containing 20 μL of TBS (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl).

*MALDI-TOF Analysis of Cross-Linked Peptide.* The eluted samples from the avidin resin were further washed and concentrated using a pipet with C18 chromatographic media ($ZipTip_{C18}$ pipet tips; Millipore Corp., Billerica, MA) following the manufacturer's directions and resuspended in 60% acetonitrile−40% water (0.1% TFA). For MALDI-TOF analysis α-CHCA (20 mg/mL) (in 50:50 acetone−2-propanol) was used as the matrix. The samples (0.5 μL) were either mixed with 0.5 μL of matrix before spotting on the target or 1.0 μL of matrix was spotted and allowed to dry before applying 1.0 μL of samples (*24*). MADLI-TOF spectra were acquired on a Bruker Daltonics Microflex using the reflector method. Masses were calculated using PROWL peptide mass prediction tools (*32*) and also based on the chemistry of the DOPA cross-linking (*19*).

## RESULTS

*Synthesis and Characterization of Bio-DOPA-α-factor.* The synthesis of analogues of α-factor containing 3,4-dihydroxyphenylalanine (DOPA) in place of $Tyr^{13}$ was carried out using automated solid-phase synthesis on a WANG resin. The intermediate Fmoc-protected peptide was obtained in high yield and purity and was used in the hydroxysuccinimiude-mediated addition of biotinylamidocaproate (*14*). The final peptides used in bioassays and cross-linking studies were virtually homogeneous as judged by gradient HPLC and had the expected molecular masses. The structures and MALDI-TOF analysis of α-factor and Bio-DOPA-α-factor are shown in Figure 1. The MS/MS spectra of the peptides are shown in Figure 2. On both the native and Bio-DOPA-α-factor spectra, 11 out of the 13 *b* ion fragments and 10 out of the 13 *y* ion fragments were observed. In the MS/MS analysis of the Bio-DOPA-α-factor a mass shift of about 340 Da was observed in fragment ions *b*7 to *b*12 due to the tagging of biotinylaminocaproate (340.47 Da) onto $Lys^7$. A mass shift of about 16 Da was observed in *y*3 to *y*6 due to the replacement of $Tyr^{13}$ with $DOPA^{13}$ (DOPA has an OH (17 Da) replacing the H (1 Da) on $Tyr^{13}$; see Figure 1A). In addition, a mass shift of 356 Da was observed in *y*7−*y*13 due to both the tagging of biotinylaminocaproate onto $Lys^7$ and the replacement of Tyr ($Y^{13}$) with $DOPA^{13}$. The overall ion fragments observed covered the entire 13 amino acids in both the native and Bio-DOPA-α-factor.

*Bioactivity and Binding of Bio-DOPA-α-factor.* Previously, we reported [$Bpa^{13}$]-α-factor was useful for photoaffinity labeling of Ste2p (*14, 15*). The diphenyl ketone side chain in Bpa is considerably larger than the phenol side chain of the native α-factor. This may have resulted in a 12-fold lower binding affinity and reduced potency of the pheromone analogue. Because of the structural similarity of DOPA to Tyr (see Figure 1), we believed that DOPA would make an equal or better replacement than Bpa. Biotin was tagged onto $Lys^7$ for detection purposes (*16, 33*). The replacement of DOPA at position 13 ([$DOPA^{13}$]-α-factor) resulted in a biological activity of 83% and binding affinity of 77% compared to that of the native α-factor (Figure 3). [$K^7$(biotinylaminocaproate)]α-factor ([$Lys(Bio)^7$]α-factor or WHWLQLK(biotinACA)PGQPNleY-OH) exhibited a biological activity of 39% and binding affinity of 13% compared to wild-type ligand. For Bio-DOPA-α-factor about a 3-fold (35%) and 10-fold (10%) reduction were observed in the biological activity and binding affinity, respectively (Figure 3). Thus, the reduction in the activities of Bio-DOPA-α-factor was mainly due to tagging with biotin and not the DOPA replacement. We concluded that DOPA at position 13 did not significantly alter the interaction of the C-terminus of α-factor with Ste2p.

*Bio-DOPA-α-factor Cross-Links into Ste2p.* Membranes from cells expressing Ste2p (His- and FLAG-tagged) were incubated with Bio-DOPA-α-factor and cross-linked by periodate oxidation. Cross-linking was carried out in the absence or presence of native α-factor, an α-factor antagonist, a synergist, or BSA as controls to evaluate the specificity of the cross-linking. The antagonist binds to Ste2p with high affinity, and the synergist does not bind to Ste2p yet influences its biological activity (*28*). Following incubation with the peptide probes, the membranes were treated with sodium periodate to oxidize DOPA and initiate cross-linking. The reaction was quenched with DTT. Membrane proteins were resolved on SDS−PAGE, blotted, and then probed with antibody to detect Ste2p (Figure 4A) or neutravidin-HRP to detect Bio-DOPA-α-factor (Figure 4B). A band (~52−54 kDa) corresponding to the size of Ste2p (52 kDa) was observed in all lanes (Figure 4A). All of the samples loaded had similar amounts of Ste2p based on the anti-Step antibody blots. When the immunoblots were probed with neutraavidin-HRP (Figure 4B), a distinct band at 54 kDa was detected in the lane where Bio-DOPA-α-factor was oxidized in the presence of Ste2p. This band was absent in samples that were not treated with Bio-DOPA-α-factor, indicating that the ~54 kDa is a Ste2p-Bio-DOPA-α-factor (52 + 2 kDa) cross-linked product. The cross-linking was nearly eliminated in the presence of excess (100-fold) α-factor and significantly reduced in the presence of a 100-fold excess of the α-factor antagonist. In the presence of BSA or synergist no effect on the level of cross-linked product was observed.

As a control for the cross-linking of Bio-DOPA-α-factor into Ste2p, membranes prepared from cells devoid of Ste2p

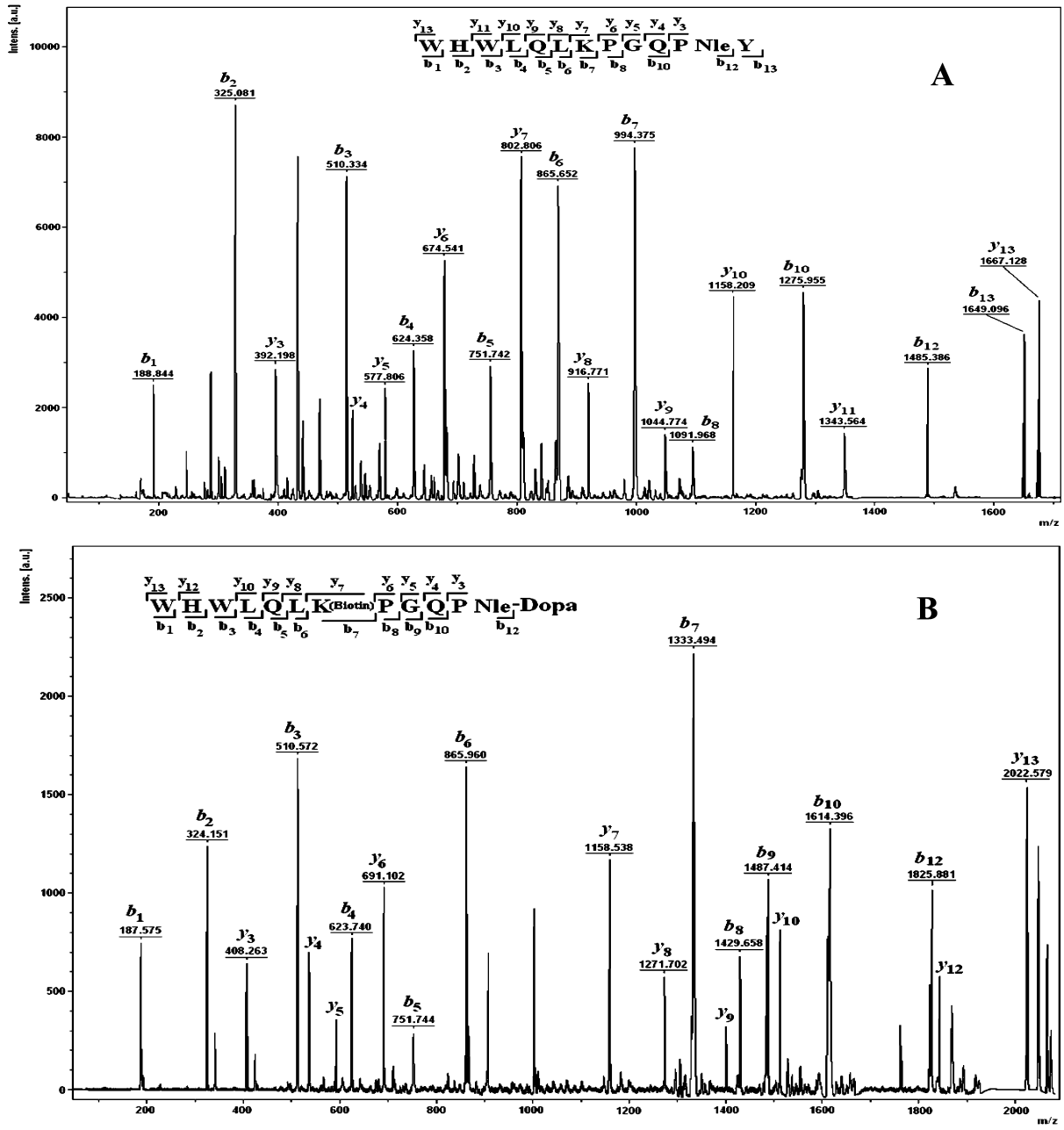


FIGURE 2: MALDI postsource decay spectra of native (A) and Bio-DOPA (B) α-factor peptides. Inserts are one-letter amino acid residue abbreviations of the peptides labeled with the identified ion types. The peaks that correspond to the theoretical fragmentation ion sizes and the type of ions are labeled accordingly. (A) In the native α-factor spectrum, 11 and 10 out of 13 of *b* and *y* ions were observed, respectively. (B) For the Bio-DOPA-α-factor, 11 and 10 out of 13 of *b* and *y* ions were also observed, respectively. A mass shift of 356 DA was observed due to the tagging of biotinylaminocaproate onto $K^7$ and the replacement of Tyr ($Y^{13}$) with $DOPA^{13}$.

were mixed with Bio-DOPA-α-factor and treated with sodium periodate under the same conditions. No detectable signal was observed on the anti-Ste2p antibody blots in the Ste2p-devoid samples (Figure 5A); however, a weak signal (~52 kDa) similar in size to that of Ste2p was detected on blots of membranes probed with neutravidin-HRP (Figure 5B) that was the result of nonspecific association of the Bio-DOPA-α-factor with a membrane protein(s) in a Ste2p-independent manner. To remove Bio-DOPA-α-factor that was noncovalently associated with Ste2p, Bio-DOPA-α-factor treated membranes from cells expressing Ste2p and lacking Ste2p were mixed with a His-HC nickel resin after the cross-linking reaction. A distinct band (~54 kDa) corresponding to a cross-linked product of Ste2p and Bio-DOPA-α-factor was observed in enriched samples (lanes labeled "P") from cross-linked membranes containing Ste2p on both the anti-Ste2p antibody and neutravidin-HRP blots (Figure 5). However, this band was absent in the Ste2p deletion mutant (Ste2pΔ, lane "P"). Thus, Bio-DOPA remained linked to Ste2p even after the harsh treatment for purifying Ste2p, indicating that a stable cross-link existed between Ste2p and Bio-DOPA-α-factor. Moreover, the Bio-Dopa-α-factor that associated with non-Ste2p proteins could be completely removed by this treatment.

*$Phe^{55}$—$Met^{69}$ Cross-Linked to Bio-DOPA.* The cross-linked Ste2p samples were eluted from the His-HC nickel resin and treated with CNBr to fragment Ste2p. The digest was mixed with avidin beads to capture the CNBr fragment of Ste2p cross-linked to Bio-DOPA-α-factor. Samples were eluted from the avidin beads with 0.1 M glycine, pH 2.5, and were

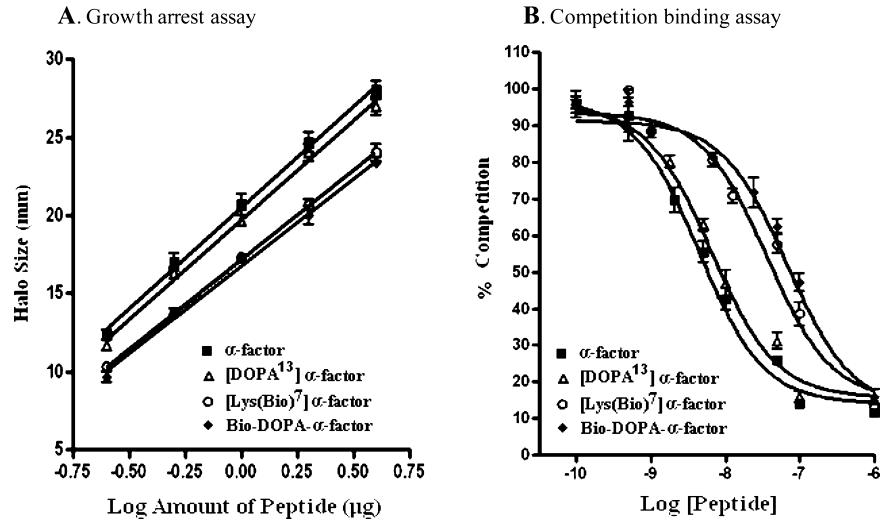


FIGURE 3: Biological and binding assays of DOPA-containing α-factor analogues. (A) Growth arrest assay to determine the ability of the peptides to activate α-factor receptor, Ste2p. (B) The binding of the peptides was determined by competition binding with [$^3$H]-α-factor for Ste2p. The assays are described in detail in the Experimental Procedures.

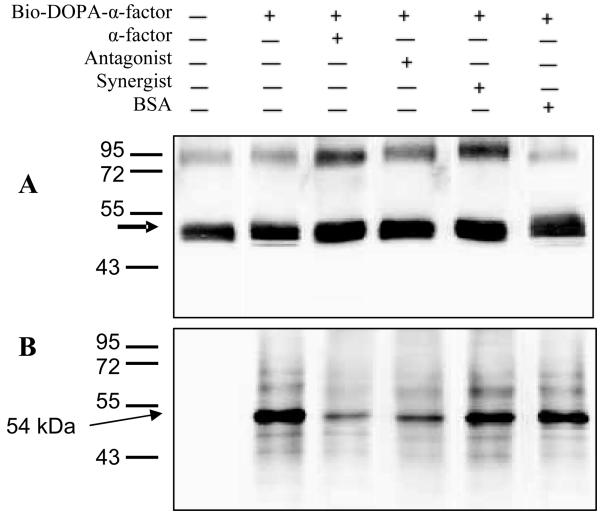


FIGURE 4: Bio-DOPA-α-factor chemical cross-linking into Ste2p. Cell membranes containing Ste2p were incubated with Bio-DOPA-α-factor alone or with Bio-DOPA-α-factor in the presence of 100-fold wild-type α-factor, antagonist, synergist, or BSA. The membranes were treated with sodium periodate to oxidize DOPA and initiate cross-linking, and the reaction was quenched with DTT. The samples were resolved on SDS−PAGE and probed with an antibody against Ste2p (A) and with neutravidin-HRP conjugate to detect the biotin tag on Bio-DOPA-α-factor (B).

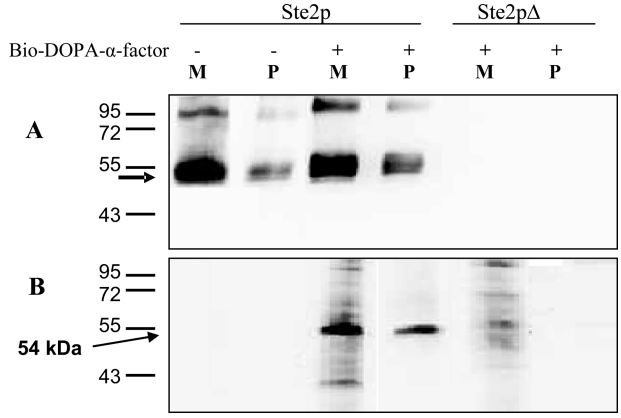


FIGURE 5: Immunoblot analysis of Bio-DOPA-α-factor reacted with membranes lacking Ste2p. Cell membranes containing and lacking Ste2p were treated with Bio-DOPA-α-factor for cross-linking. The samples were separated on SDS−PAGE and probed with an antibody directed against Ste2p (A) and with neutravidin-HRP conjugate (B). M indicates membrane extract; P indicates enriched samples from His-HC nickel resin.

concentrated using a Millipore ZipTip to remove detergent and compounds that might interfere with mass spectrometry analysis (*34*). MALDI-TOF analysis (Figure 6A) showed that the eluted sample contained two peaks, 3497.01 and 3815.21 Da, corresponding to a CNBr-Ste2p fragment, $F^{55}-M^{69}$ (1473.81 Da, assuming a homoserine lactone terminus) cross-linked to Bio-DOPA-α-factor (2021.43 Da) (total calculated mass = 3494.24; observed mass = 3497.01), and a CNBr-Ste2p fragment $F^{55}-M^{71}$ (1792.22 Da, assuming a homoserine lactone terminus) cross-linked to Bio-DOPA-α-factor (2021.43 Da) (total calculated mass = 3812.65; observed mass = 3815.21), respectively. The calculated masses were based on M +1H (mass spectrometric ionization) and the loss of 2H from the cross-linking reaction (see Figure 8 for mechanism). Two additional peaks (2134 and 2291) were also found in the cross-linked sample and a sample eluted from avidin beads mixed with CNBr-treated Ste2p that had not been subjected to cross-linking (Figure 6B). Thus, these additional peaks were associated with peptides that were copurified with Ste2p during the enrichment with the His-HC nickel resin and were also retained by the avidin beads.

*Bio-DOPA Cross-Links to Cys*$^{59}$. It has been shown previously that the sulfhydryl group of cysteine is able to covalently cross-link to DOPA upon sodium periodate-mediated oxidation (*19*). Since Cys is part of the Ste2p fragment detected, we carried out a cross-linking experiment with a cysteine-less Ste2p (Cys59Ser, Cys252Ser). Membranes were prepared from cells expressing Ste2p wild type, the cysteine-less mutant, or no Ste2p. The cross-linking and enrichment of Ste2p were carried out as described above. The immunoblot analysis (Figure 7B) showed that cross-linking of Bio-DOPA-α-factor into the cysteine-less Ste2p was 83% less efficient in comparison to the wild-type receptor, although similar levels of Ste2p signal were detected in both the wild-type and Cys-less samples (Figure 7A). We also showed that Bio-DOPA-α-factor cross-linked to a

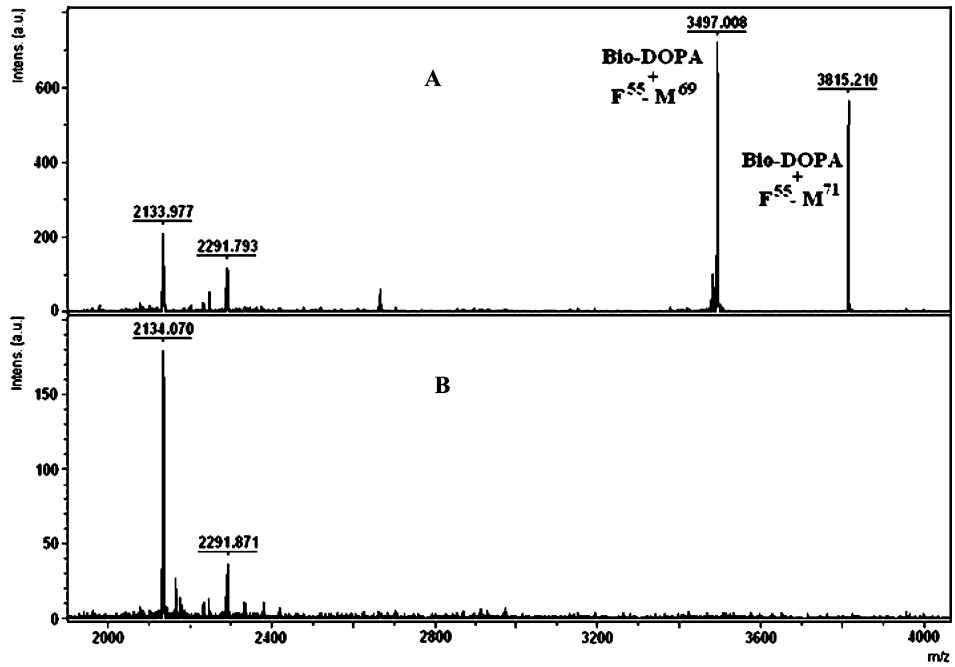


FIGURE 6: MALDI-TOF analysis of CNBr digested cross-linked Ste2p. Cross-linked Ste2p samples eluted from His-HC nickel gel were digested with CNBr and then mixed with avidin resin. (A) Spectrum for samples eluted from avidin resin cross-linked Ste2p samples. The 3497 and 3815 Da peaks correspond to Ste2p fragments $F^{55}-M^{69}$ and $F^{55}-M^{71}$ cross-linked to Bio-DOPA, respectively. (B) Spectrum for samples eluted from avidin resin from CNBr digested Ste2p without cross-linking. Two major peptides were detected which were also observed in cross-linked Ste2p samples (A).

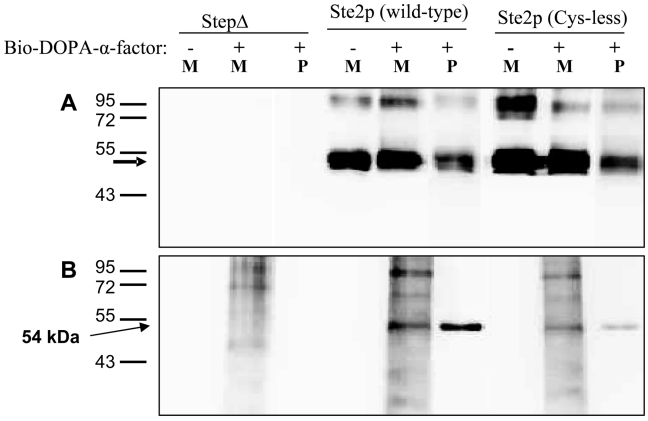


FIGURE 7: Cross-linking of Bio-DOPA-α-factor to Cys-less Ste2p. Membranes from cells expressing wild-type, Ste2p-C59S, or lacking Ste2p were incubated with Bio-DOPA-α-factor and periodate reagent. A portion of the cross-linked membranes was mixed with His-HC nickel to purify Ste2p. The various fractions were analyzed by polyacrylamide electrophoresis, and the gels were blotted and probed with anti-Ste2p antibody (A) or with (B) neutravidin. M indicates membrane extract; P indicates enriched samples from His-HC nickel resin.

Cys59Ala mutant at almost the same low level as that of the Cys59Ser mutant.

## DISCUSSION

Cross-linking studies followed by mass spectrometry analysis of the product have become increasingly used to elucidate contacts between ligands and their cognate receptors (*35*). In addition, a few studies have pinpointed a residue-to-residue interaction between a peptide and its GPCR using photoaffinity labeling (*36*−*40*). Ste2p, the α-factor pheromone receptor of *S. cerevisiae*, has been used as a model for interactions between small peptides and GPCRs. Photochemical cross-linking, site-directed mutagenesis, and double mutant cycle analysis approaches concluded that residues 1, 3, 10, and 13 of α-factor are involved in binding to Ste2p (*13, 41*−*44*). Several regions of Ste2p that contact α-factor were previously identified (*14*−*17, 24, 45*). However, in no case was the exact residue−residue interaction between α-factor and Ste2p determined.

Periodate-mediated cross-linking of DOPA has been shown to result in a stable covalent cross-link between interacting polypeptides (*19, 20*). DOPA-mediated cross-linking was previously shown to be efficient and specific as only one protein was labeled using a mixture of ten proteins (*18*). The methodology revealed contacts between Rpt6p/Sug1p and Rpt4p/Sug2p proteins and the Gal4 transcriptional activation domains (*21*) and was also used to investigate peptoid−inhibitor interactions with the 19S regulatory particle of the 26S proteasome (*22*). However, none of these studies defined the contact points between cross-linked molecules. The present study was conducted to explore the utility of 3,4-dihydroxyphenylalanine (DOPA) oxidative chemical cross-linking in analyzing hormone−receptor interactions. The interaction of GPCR, Ste2p, and its cognate peptide ligand, α-factor, was used as the test system.

The results presented in this paper show that replacement of $Tyr^{13}$ by DOPA in α-factor results in a high-affinity, potent agonist (Figure 3). Comparisons with control peptides allow the conclusion that the DOPA moiety in the Bio-DOPA-α-factor peptide used in cross-linking studies likely binds similarly to the phenol side chain in the native peptide. Thus reaction of this moiety with groups on the receptor should lead to information relevant to the natural ligand.

DOPA chemical cross-linking studies carried out using membranes prepared from cells expressing Ste2p showed that Bio-DOPA-α-factor cross-linked into Ste2p (Figures 4 and 5). When the cross-linking was carried out using membranes

lacking Ste2p under the same conditions, only a small signal was observed, and this was totally removed by treatment with an affinity column that recognized Ste2p (Figure 5B). The cross-linking of Bio-DOPA-α-factor to Ste2p was prevented by a 100-fold molar excess of native α-factor and highly reduced by a lower affinity α-factor antagonist (*28*), but it was not influenced by the presence of BSA (Figure 4). These results showed that Bio-DOPA-α-factor binds and cross-links to the ligand binding site in Ste2p and that it could be used to determine the contact between position 13 of α-factor and this GPCR.

The strong interaction between avidin and biotin has been very useful in affinity purification of biotinylated peptides (*34*) and together with MS analysis is widely used for detection of cross-linked products with high sensitivity and accuracy (*46*). We reported previously on the purification and MS analysis of a biotinylated α-factor cross-linked to a portion of Ste2p (*16*). We applied this approach to analyze the cross-linking between Bio-DOPA-α-factor and Ste2p. The cross-linked Ste2p samples were fragmented with cyanogen bromide (CNBr), and the biotinylated fragments were captured by affinity purification. MALDI-TOF analysis of the biotinylated CNBr fragments from cross-linked Ste2p showed two major peaks, 3497 and 3815 Da (Figure 6A). In addition, two other peaks (2134 and 2291 Da) were detected which were also detected in samples from un-cross-linked CNBr-treated Ste2p samples (Figure 6B). These two minor peaks (2134 and 2291 Da) were due to proteins that were copurified with Ste2p during the enrichment with the His-HC nickel resin as observed on silver and Coomassie-stained SDS−PAGE gels (data not shown). Since the two major peaks (3497 and 3815 Da) were only observed in the cross-linked Ste2p samples, we concluded that these fragments are composed of parts of Ste2p cross-linked to Bio-DOPA-α-factor. Examination of the predicted sizes of CNBr fragments of Ste2p and the peaks detected revealed that the peak 3497.01 Da corresponded to a CNBr-digested Ste2p fragment, $Phe^{55}$−$Met^{69}$ (1473.81 Da) cross-linked to Bio-DOPA (2021.43 Da), whereas the 3815.21 Da peak corresponded to a CNBr-digested Ste2p fragment $Phe^{55}$−$Met^{71}$ (1792.22 Da) cross-linked to Bio-DOPA (2021.43 Da). The approximately 3 Da difference between both experimental peaks and the theoretical values was due to averaging the observed isotopic mass of the peptides (*32*). On the basis of this result and previous studies showing that [$Bpa^{13}$]-α-factor analogue cross-linked to residues $Phe^{55}$−$Arg^{58}$ of Ste2p, we suggest that position 13 of α-factor interacts with a residue(s) within $Phe^{55}$−$Met^{69}$.

Previous investigations determined that the ϵ-amino of lysine, the imidazole of histidine, and the thiol of cysteine were capable of attacking the *o*-quinone obtained on periodate oxidation of DOPA (*19*). Based on these findings, the only nucleophilic side chain within the residues $Phe^{55}$−$Met^{69}$ ($^{55}$FGVRCGAAALTLIVM$^{69}$) that could attack the *o*-quinone intermediate is $Cys^{59}$. To efficiently couple to DOPA, the thiol group of cysteine and DOPA must be in close proximity (*19*). Thus our results suggest that $DOPA^{13}$ of Bio-DOPA cross-links to $Cys^{59}$ of Ste2p and that residue 59 of Ste2p and residue 13 of the α-factor ligand must be close proximity in the ligand-bound state of the receptor.

To investigate whether $DOPA^{13}$ of Bio-DOPA-α-factor cross-linked to $Cys^{59}$ of Ste2p, experiments were repeated

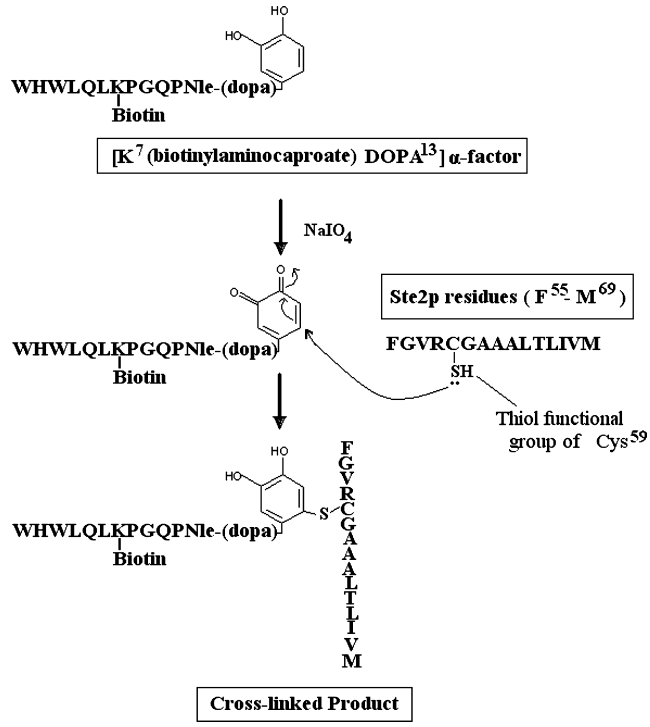


FIGURE 8: Proposed mechanism of oxidative chemical cross-linking reaction of DOPA at position 13 of Bio-DOPA-α-factor and the $Cys^{59}$ of Ste2p. DOPA is oxidized to an intermediate *o*-quinone which is then attacked by the thiol functional group of $Cys^{59}$ to form a stable covalent cross-link according to ref *19*.

using membranes containing cysteine-less Ste2p mutant. This mutant receptor has similar activities as the wild-type receptor (*23*). Immunoblotting analysis (Figure 7) showed that cross-linking of Bio-DOPA-α-factor into the cysteine-less Ste2p mutant was over 80% less efficient compared to wild type. This experiment was repeated at least three times, and similar results were obtained. We also changed Cys to Ala at position 59 while maintaining Cys at position 252. This construct showed a similar reduction in cross-linking, indicating that the majority of the cross-linked product involved Cys59 (data not shown). Thus, the substitution of cysteine with serine or alanine at position 59 of Ste2p reduced the cross-linking between Bio-DOPA-α-factor and Ste2p, supporting the conclusion that the thiol functional group of $Cys^{59}$ was cross-linked to Bio-DOPA-α-factor and that $Cys^{59}$ is in close proximity with position 13 of α-factor. The proposed mechanism for the cross-linking of Bio-DOPA-α-factor to $Cys^{59}$ of Ste2p follows a reaction scheme suggested by the Kodadek group (*22*) and is outlined in Figure 8. The minor amount of cross-linking still observed (about 20% of the wild-type level) may be due to other nucleophiles in proximity to position 59 of Ste2p. Potential candidates are Arg58, His94, and Lys100 according to the model of Ste2p based on the rhodopsin X-ray structure (*10*).

As stated above, the significant reduction in cross-linking found with the Cys-less receptor is consistent with our conclusion that Cys59 is the contact point of $Tyr^{13}$ and Ste2p. Previously, we concluded that [$Bpa^{13}$]-α-factor cross-linked to the F55-R58 fragment of Ste2p using photoactivation approaches (*14*). To probe this region further, site-directed single Ala mutations were constructed on residues 54−58 in Ste2p (data not shown). Ste2p mutants R58A, R58D, and R58E exhibited a large increase in the $K_d$ for binding [$^3$H]-

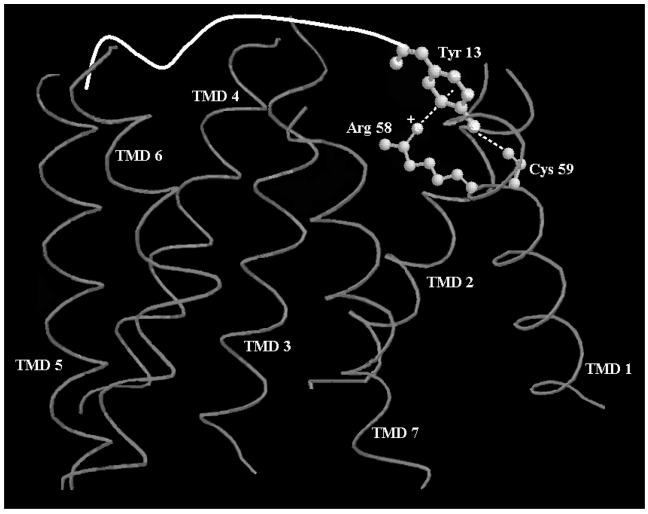


FIGURE 9: Model of the interactions of position $Tyr^{13}$ of α-factor with Arg58 and Cys59 residues of Ste2p. The $Tyr^{13}$ side chain phenyl ring is shown as involved in a cation−$\pi$ interaction with the Arg58 guanidinium moiety, and the phenolic OH forms a hydrogen bond with the Cys59 sulfhydryl group. The transmembrane domains (TMD) are labeled, respectively.

α-factor. The other mutant receptors had a similar or slightly reduced binding affinity compared to that of the wild-type receptor. Photo-cross-linking of these Ala mutants to [$Bpa^{13}$,$K^{7}$-(biotinylamidocaproate),$Nle^{12}$]-α-factor showed that cross-linking to the R58A mutant was highly diminished, but cross-linking to the F55A, G56A, and V57A mutants was not greatly affected. These cross-linking and site-directed mutagenesis results suggested that position 13 of α-factor interacted with R58 of Ste2p. The finding that [$Bpa^{13}$]-α-factor and [$DOPA^{13}$]-α-factor cross-link to Arg58 and Cys59, respectively, provides strong evidence that the carboxyl-terminal residue of the pheromone binds to this region of Ste2p. It seems reasonable that the $Tyr^{13}$ side chain is involved in a cation−$\pi$ interaction with the Ste2p-Arg58 guanidinium moiety and a hydrogen bond with the Ste2p-Cys59 sulfhydryl group (Figure 9).

At present the most detailed information pertaining to ligand binding sites of GPCRs comes from X-ray analysis of rhodopsin and $\beta$-adrenergic receptors (*11, 48*−*51*). These proteins bind retinal and inverse agonists, respectively, at sites located primarily in the TM core of these GPCRs. No high-resolution information is available on GPCRs that bind medium-sized peptide hormones; most of our knowledge on binding sites is inferred from biochemical data. The cross-linking information on α-factor−Ste2p interactions suggests that the carboxyl terminus of the tridecapeptide ligand contacts two polar residues (Arg58 and Cys59) that are near the extracellular end of TM1 of Ste2p (Figure 9). Double mutant cycle analyis (*42*) previously showed that $Trp^{1}$ and $Trp^{3}$ of α-factor interacts strongly with N205 and Y266 residues that are located near the extracellular ends of TM5 and TM6, respectively (*17, 52*). Finally, the center of α-factor is thought to face toward the extracellular loop regions of Ste2p (*17, 33*). Thus, in contrast to covalently bound retinal and small biogenic amines, the α-factor tridecapeptide interacts primarily with the ends of TM domains and loops of Ste2p during activation of its GPCR. These interactions are consistent with the prevailing belief that the binding site for peptide ligands to their GPCRs involves ectodomains (*53, 54*).

Binding of α-factor is thought to elicit a significant conformational change in EL1 that is related to receptor activation. Intriguingly, the extracellular loop of rhodopsin is also believed to move during light activation of this GPCR, and the signaling role of extracellular loops in other class A GPCRs is just starting to be revealed as well (*55*). Ste2p is a class D GPCR, and rhodopsin is a class A GPCR. If extracellular loops are involved in activation of such evolutionary distinct receptors, it is not unreasonable to suggest that movements of extracellular loops may be an important, perhaps general, aspect of GPCR signal transduction. Evidently, different classes of GPCRs actuate these movements by distinct molecular interactions.

In conclusion, we have synthesized and characterized an α-factor analogue (Bio-DOPA-α-factor) with DOPA replacing $Tyr^{13}$ and biotin tagged onto $Lys^{7}$ and shown that it linked covalently into Ste2p by periodate-mediated DOPA cross-linking. Mass spectrometric analyses of the cross-linked fragments demonstrated that Bio-DOPA cross-linked into residues $Phe^{55}$−$Met^{69}$ of Ste2p. Replacing cysteine at position 59 with serine or alanine drastically reduced the cross-linking with Bio-DOPA-α-factor. Therefore, it is reasonable to conclude that $Tyr^{13}$ of α-factor is in close proximity to $Cys^{59}$ of Ste2p when α-factor is bound to Ste2p. Our results represent the first determination of a residue-to-residue contact point between α-factor and Ste2p and support the use of oxidative cross-linking to investigate peptide−GPCR interactions.

## ACKNOWLEDGMENT

We thank Larry Thompson for advice on the mass spectrometry studies and James Konopka for the generous gift of antibody to Ste2p.

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BI802061Z