Redirecting RiPP biosynthetic enzymes to proteins and backbone-modified substrates

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I. Materials and general information

Safety Statement
No unexpected or unusually high safety hazards were encountered during the completion of this work.

Commercial materials. Acetonitrile, Tris base, HEPES, sodium chloride (NaCl), and agar were purchased from Fisher Scientific (Hampton, NH). Trifluoroacetic acid (TFA), bromophenol blue, imidazole, riboflavin, carbenicillin, and adenosine 5'-triphosphate (ATP) were purchased from Sigma Aldrich (St. Louis, MO). Glycerol and sodium dodecyl sulfate (SDS) were purchased from American Bioanalytical (Natick, MA). Glycine and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Thermo Scientific (Fairlawn, NJ). Magnesium chloride ($MgCl_2$) and sodium phosphate monobasic were purchased from Avantor Sciences (Radnor, PA). LC-MS grade formic acid was purchased from RICCA Chemical Company (Arlington, TX). 2-mercaptoethanol (βME) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Kanamycin was purchased from MP Biomedical LLC (Solon, OH). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from EMD Millipore (Burlington, MA). Bicine was purchased from J&K Scientific (San Jose, CA). Riboflavin-5'-phosphate (FMN) was purchased from Spectrum Chemical MFG Corp (Gardena, CA). V8 protease (endoproteinase Glu-C) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

II. Peptide synthesis

Reagents. All purchased reagents were used without further purification. Fmoc-Gly-Wang resin and standard fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were purchased from Novabiochem (San Diego, CA). Diethyl ether, trifluoroacetic acid (TFA), ethanedithiol (EDT), piperidine, benzoic acid (Bz), and 1-hydroxybenzotriazole (HOBt) were purchased from Sigma Aldrich (St. Louis, MO). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-2-aminobenzoic acid (Fmoc-2-Abz-OH), and Fmoc-3-amino-2,2-dimethyl-propionic acid (Fmoc-β^2-Dimethyl-OH) were purchased from Chem-Impex International Inc. (Wood Dale, IL). Dichloromethane (DCM) dimethylformamide (DMF), and 2 M diisopropylethylamine (DIPEA) in N-methyl pyrolidinone (NMP) were purchased from Fisher Scientific (Hampton, NH). Triisopropylsilane (TIPS), 2,3,5,6-tetrafluorobenzoic acid (TFBz), and 2-amino-5-methoxybenzoic acid (5-Methoxy-2-Abz) were purchased from Acros Organics (Carlsbad, CA). (1S,2S)-Fmoc-2-amino-1-cyclopentane carboxylic acid (Fmoc-ACPC-OH) and (1S,2S)-Fmoc-2-amino-1-cyclohexane carboxylic acid (Fmoc-ACHC-OH) were purchased from AnaSpec, Inc. (San Jose, CA). Fmoc-L-β^3-Homoisoleucine (Fmoc-β^3-HIle-OH) was purchased from PepTech Corporation (Burlington, MA). Coumarin-3-carboxylic acid (Coumarin) was purchased from Combi-Blocks, Inc. (San Diego, CA). 3-aminopyridine-4-carboxylic acid (APy) was purchased from Accela (San Diego, CA). Bromotripyrrolidinophosphonium hexafluorophosphate (PyBroP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 2,6-lutidine was purchased from J&K Scientific (San Jose, CA).

Solid phase peptide synthesis. Peptides 1a-i and 4a-d were synthesized on a 50 μmol scale using Fmoc chemistry and Fmoc-Gly-Wang resin (0.61 mmol/g substitution, 100 - 200 mesh) on a Biotage® Initiator+ Alstra from Biotage (Charlotte, NC). Resin was first swollen in 4.5 mL of DMF for 20 minutes at 70°C. The Fmoc protecting group was then removed by two room temperature treatments (3 minutes followed by 10 minutes) with 4.5 mL of deprotection solution (0.1 M HOBt in 20% (v/v) piperidine in DMF). Excess deprotection solution was then removed by 4 washes with 4.5 mL of DMF. Coupling reactions were performed by adding 5 equivalencies of amino acid, HOBt, and HBTU (from 200 mM stocks in DMF) and 10 equivalencies of DIPEA (from a 2 M stock in NMP) to the deprotected resin for a final resin-bound peptide concentration of 12.5 mM. Standard coupling reaction conditions were 75°C for 5 minutes. To minimize racemization, cysteine was coupled at 50°C for 15 minutes. Excess coupling reagents were removed by 4 washes with 4.5 mL of DMF. Once the synthesis was complete, the resin-bound peptide was dried under nitrogen and removed from the resin by treatment with 3 mL of cleavage cocktail (92.5% TFA, 2.5% water, 2.5% TIPS, and 2.5% EDT) for 1 hour at room temperature. Cleaved peptide was precipitated in 40 mL of diethyl ether chilled to -80°C and lyophilized before purification via HPLC.

Peptides 4e-g were synthesized as described above for peptides 1a-i and 4a-d with the following modifications. Following coupling of the non-α-amino acid monomer, the resin-bound peptide was transferred to a fritted glass reactor. Coupling of cysteine to the non-α-amino acid monomer was adapted from previous reports.[1] Briefly, 5 equivalencies of amino acid and PyBroP (from 400 mM stocks in DCM:DMF (9:1)) and 15 equivalencies of 2,6-lutidine were added to the deprotected resin for a final resin-bound peptide concentration of 33 mM. The coupling reaction was then carried out at room temperature overnight (at least 16 hours). Excess coupling reagents were removed by 4 washes with 4.5 mL of DMF. The Fmoc protecting group was then removed as described above. Coupling reactions for the N-terminal isoleucine residue were performed by adding 5 equivalencies of amino acid, HOBt, and HBTU (from 200 mM stocks in DMF) and 10 equivalencies of DIPEA (from a 2 M stock in NMP) to the deprotected resin for a final resin-bound peptide concentration of 12.5 mM. The coupling reaction conditions were room temperature for 1 hour. The N-terminal Fmoc protecting group was then removed and the completed peptide cleaved from the resin as described above before purification via HPLC.

HPLC purification. HPLC purification was performed on an Agilent 1260 Series HPLC system equipped with a UV diode array detector and an 1260 Infinity fraction collector using a semi-preparative reversed-phase C18 column (YMC Group, Triart C18, 10 x 150 mm, 5-Micron). The mobile phase for HPLC was water with 0.1% (v/v) trifluoroacetic acid (solvent A) and acetonitrile with 0.1% (v/v) trifluoroacetic acid (solvent B). Peptides were eluted at a flow rate of 4 mL/min using a linear solvent gradient from 5 - 95% acetonitrile in water over 30 minutes. Peptides were collected based on their absorbance at 280 nm.

Mass spectrometry of purified peptides. LC-MS analysis of each peptide was performed on an Agilent 1290 Infinity II HPLC connected to an Agilent 6530B QTOF AJS-ESI. The mobile phase for LC-MS was water and acetonitrile with 0.1% (v/v) formic acid and a flow rate of 0.7 mL/min. Each peptide was injected onto an Eclipse XDB C-18 column (2.1 x 50 mm, 1.8-Micron, room temperature, Agilent) and separated using a linear gradient from 5 to 95% acetonitrile over 4.5 minutes after an initial hold at 5% acetonitrile for 0.5 minutes. The following parameters were used during acquisition: Fragmentor voltage 175 V, gas temperature 300ºC, gas flow 8 L/min, sheath gas temperature 350ºC, sheath gas flow 11 L/min, nebulizer pressure 35 psi, skimmer voltage 65 V, Vcap 3500 V, 1 spectra/s.

III. Enzyme expression, purification, and characterization

A. MicD-F expression plasmid and translation product

The plasmid used to express MicD-F (pJExpress411-MicD-F) was graciously provided by Professor James Naismith (University of Oxford).[2] The translation product encoded by this plasmid is the full sequence of MicD (heterocyclase from *Microcystis aeruginosa,* Uniprot ID: A8Y998) preceded by five repeats of a **Gly-Ala spacer**, residues **Thr18 to Ala37 from PatE** (Uniprot ID: A0MH79), a **TEV protease recognition site**, and an N-terminal **6xHis** purification tag. The full sequence of the translation product is provided below.

MS**HHHHHH**DYD**ENLYFQG**SRL**TAGQLSSQLAELSEEALGDAGAGAGAGAGA**KLMQSTPLLQ
IQPHFHVEVIEPKQVYLLGEQANYALTGQLYCQILPLLDGQHSREQIVEKLDGEVPSEYIDYVLDR
LAEKGYLTEAAPELSSEVAAFWSELGIAPPVAAEALRQSVTLTPVGNISEVTVAALTTALRDIGISV
QTPTEAGSPTALNVVLTDDYLQPELAKINKQALESQQTWLLVKPVGSVLWLGPVFVPGKTGCW
DCLAHRLRGNREVEASVLQQKQAQQQRNGQSGSVIGCLPTARATLPSTLQTGLQFAATEIAKWI
VKHHVKATAPGTVFFPTLDGKIITFNHTVIDLKSHVLVRRSQCPSCGDRQILHRQGFEPVKLVSR
RKHFTHDGGHRAFTPEQTVQKYQHLVSPITGVVTELVRLTDPANPLVHTYKAGHAFGSATTLRG
LRNTLKYKSSGKGKTDIQSRASGLCEAIERYSGIFQGDEPRKRATLAELGDLALHPESLLYFSNT
QYANREELNAQGSAAAYRWIPNRFDVSQAIDWTPVWSLTEQKHKYVPTAFCYYGYPLPEEQRF
CKADSNGNAAGNTLEEAILQGFLELVERDSIAMWWYNRIRRPAVDLSTFDEPYFVDLQQFYQQ
QNRELWVLDVTADLGIPAFAGFSRRTVGTSERISIGFGAHLDPTIAILRALTEVSQVGLELDKIPDD
KLDGESKDWMLNVTVENHPWLAPDPSVPMKTASDYPKRWSDDIHTDVMNCVKTAQTAGLEV
MVLDQTRPDIGLNVVKVIIPGMRTFWTRFGQGRLYDIPVKLGWLDAPLAEEELNQTNIPF

B. MicD-F expression and purification

A starter culture of 5 mL of Miller's LB Broth (AmericanBio, catalog # AB01201) containing 50 µg/mL kanamycin was inoculated with a single colony of *E. coli* BL21 (DE3) harboring the pJExpress411-MicD-F plasmid and grown overnight at 37ºC with shaking at 200 rpm. The starter culture (5 mL) was used to inoculate a 500 mL expression culture of Miller's LB Broth which also contained 50 µg/mL kanamycin. The expression culture was grown at 37ºC with shaking at 200 rpm to an OD_{600} of 0.6 at which point the expression culture was induced with 1

mM IPTG, transferred to a 20°C incubator, and grown for 24 hours with shaking at 200 rpm. The expression culture was harvested by centrifugation at 4,300xg at 4°C for 20 minutes. The resulting cell pellet was suspended in 10 mL of Lysis Buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) containing 1 tablet of cOmplete, mini EDTA-free ULTRA protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell suspension was disrupted by sonication on ice (Branson Sonifier 250, 3 cycles of 30 second pulse at 30% duty cycle and microtip limit of 5 followed by 60 second pause). The cell lysate was cleared by centrifugation at 4,300xg at 4°C for 20 minutes. A gravity flow Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA) was loaded with 2 mL of packed Ni-NTA agarose (Qiagen, Germantown, MD) and equilibrated with 10 mL of Lysis Buffer. The 6xHis-tagged protein was bound to the Ni-NTA column by passing the cleared cell lysate over the column three times. Non-specifically bound proteins were removed by washing the Ni-NTA column with 10 mL of Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole pH 8.0). The 6xHis-tagged protein was eluted by washing the Ni-NTA column with 5 mL of Elution Buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole pH 8.0). The purified protein was concentrated and exchanged into Storage Buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) using Amicon Ultra-0.5 mL centrifugal filters (MilliporeSigma, Burlington, MA) with a 30 kDa molecular weight cut off according to the manufacturer's instructions. The purified protein was quantified using absorbance at 280 nm (molar extinction coefficient of 125,250 M^{-1} cm^{-1}), diluted to 500 µM using Storage Buffer, snap frozen as single-use aliquots, and stored at -80°C. The typical expression yield of MicD-F using the above protocol was 30 mg/L of *E. coli* culture.

C. ArtGox expression plasmid and translation product

The plasmid used to express ArtGox (pEHISTEVSUMO-ArtGox) was generously provided by Professor James Naismith (University of Oxford).[3,4] The translation product encoded by this plasmid is the oxidase domain of ArtG (thiazoline oxidase from *Arthrospira platensis,* Uniprot ID: H1W8K1) preceded by a **TEV protease recognition site**, a **SUMO fusion tag**, and an N-terminal **6xHis** purification tag. The full sequence of the translation product is provided below.

MGSS**HHHHHH**G**SDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFA KRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG**DIPTT**ENLYFQG**AMGRTYP FAVSLNSTIQVSTTADGYAISPANTDPGQSIAPSMVTLPAITGMGDALAHLQAGTATLQQLTQTLS AREGVEAGEQLAATLQQMGDRGWLQYAVLPLAIAEPMVESAELDLNSPHWTQAKVSLSRFAY QRSHAGGMVLESPLSKFRVKLLDWRSSAILAQLAQPQPLGWVTPPPQIGAETAYQFLNLLWAT GFLTVETEAPELKLWEFHNLLFHSRCRQGRHDYPTGDIAASLDIWDEFPVVKPPMSGHIVPLPQ LSIDAIRQRDKTLTTAIEKRASIREYDENHPITIEQLGELLYRTARIKEIYTHDAEQAELLKAQFGED FDWGELSRRPYPCGGAMYELEIYLAVRRCAGVKPGLYHYDPLNHQLAQIDAADADIQALLKDA HQSSGEQGMPQVLLMITARFGRLFRKYRSLAYALVLKHVGVLYQNLYLVATNMGLAPCALGAGD SDRFAQATGLDYVVESSVGEFMLGSL

D. ArtGox expression and purification

A starter culture of 5 mL of Miller's LB Broth (AmericanBio, catalog # AB01201) supplemented with 50 µg/mL kanamycin was inoculated with a single colony of *E. coli* BL21 (DE3) harboring the pEHISTEVSUMO-ArtGox plasmid and grown overnight at 37°C with shaking at 200 rpm.The starter culture (5 mL) was used to inoculate a 500 mL expression culture of Miller's LB Broth supplemented with 50 µM riboflavin and containing 50 µg/mL kanamycin. The expression culture was grown at 37°C with shaking at 200 rpm to an OD_{600} of 0.6 at which point it was induced with 1 mM IPTG, transferred to a 20°C incubator, and grown for 24 hours with shaking at 200 rpm. The expression culture was harvested by centrifugation at 4,300xg at 4°C for 20 minutes. The resulting cell pellet was suspended in 10 mL of Lysis Buffer (20 mM Tris-HCl, 500 mM NaCl, 50 µM flavin mononucleotide, pH 8.0) containing 1 tablet of cOmplete, mini EDTA-free ULTRA protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell suspension was disrupted by sonication on ice (Branson Sonifier 250, 3 cycles of 30 second pulse at 30% duty cycle and microtip limit of 5 followed by 60 second pause). The cell lysate was cleared by centrifugation at 4,300xg at 4°C for 20 minutes. A gravity flow Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA) was loaded with 2 mL of packed Ni-NTA agarose (Qiagen, Germantown, MD) and equilibrated with 10 mL of Lysis Buffer. The 6xHis-tagged protein was bound to the Ni-NTA column by passing the cleared cell lysate over the column three times. Non-specifically bound proteins were removed by washing the Ni-NTA column with 10 mL of Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, 50 µM flavin mononucleotide, pH 8.0). The 6xHis-tagged protein was eluted by washing the Ni-NTA column with 5 mL of Elution Buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, 50 µM flavin mononucleotide, pH 8.0). The purified protein was concentrated and exchanged into Storage Buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) using Amicon Ultra-0.5 mL centrifugal filters (MilliporeSigma, Burlington, MA) with a 30 kDa molecular weight cut off according to the manufacturer's instructions. The purified protein was quantified using absorbance at 280 nm (molar extinction coefficient of 75,290 M^{-1} cm^{-1}), diluted to 800 µM using Storage Buffer, snap frozen as single use aliquots, and stored at -80°C. The typical expression yield of ArtGox using the above protocol was 40 mg/L of *E. coli* culture.

E. Characterization of MicD-F and ArtGox

SDS-PAGE. Purified samples of MicD-F and ArtGox (4 µg) were mixed (4:1) with SDS-PAGE sample buffer (5% β-Mercaptoethanol, 0.02% bromophenol blue, 30% glycerol, 10% SDS, 250 mM Tris-HCl, pH 6.8). The protein samples were reduced and denatured by incubation at 95°C for 5 minutes. Reduced and denatured samples were separated using a 4-15% mini-PROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA) run at 120 V for 60 minutes in Tris-Glycine-SDS running buffer (3 g/L tris, 14.4 g/L glycine, 1 g/L sodium dodecyl sulfate, pH 8.3) and their molecular weights compared against Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Hercules, CA). Protein content was visualized using Coomassie stain (1 g/L Coomassie Brilliant Blue in methanol:water:acetic acid (5:4:1)) and imaged using a Bio-Rad Chemidoc MP Imaging System. Band intensities were quantified using the gel analysis tool of

FIJI.[5] Please see **Supplementary Figure 1** for SDS-PAGE and LC-MS characterization of MicD-F and ArtGox.

IV. Cloning, expression, purification, and characterization of mCherry and Rop variants

A. Design of mCherry variants

C-terminally modified substrates (mCherryC+ and mCherryC-) are variants of the mCherry sequence associated with Uniprot ID: X5DSL3. Both contained an N-terminal **6xHis** purification tag, the natural three-residue chromophore-forming sequence **MYG**, and a C-terminal extension that included a **TEV protease recognition site**, a MicD-F/ArtGox compatible **substrate**, and a **FLAG** purification tag. Internally-modified substrates (mCherry137+, mCherry174+, mCherry192+ and mCherry211+) are also variants of the mCherry sequence associated with Uniprot ID: X5DSL3. Each contained an N-terminal **FLAG** purification tag, the natural three-residue chromophore-forming sequence **MYG**, and a C-terminal **6xHis** purification tag. A MicD-F/ArtGox compatible **substrate** sequence was inserted internally on the C-terminal side of the indicated residue (137, 174, 192, or 211). The full sequences of the mCherry translation products are provided below.

mCherryC+
MHHHHHHMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTK
GGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSL
QDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDA
EVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKENLYFQGM
CAYDGDYKDDDDK

mCherryC-
MHHHHHHMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTK
GGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSL
QDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDA
EVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKENLYFQGM
AAYDGDYKDDDDK

mCherry137+
MDYKDDDDKMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS
SLQDGEFIYKVKLRGTNFPSDMCAYDGGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKL
KDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK
HHHHHH

mCherry174+

MDYKDDDDKMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS
SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDMCAY
DGGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELY
KHHHHHH

mCherry192+

MDYKDDDDKMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS
SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHY
DAEVKTTYKAKKPVMCAYDGQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK
HHHHHH

mCherry211+

MDYKDDDDKMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS
SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHY
DAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEMCAYDGDYTIVEQYERAEGRHSTGGMDELYK
HHHHHH

B. Cloning mCherry constructs into pET-32a(+) vector

The sequences encoding mCherryC+, mCherryC-, mCherry137+, mCherry174+, mCherry192+ and mCherry211+ were cloned into a pET-32a(+) plasmid as follows. Circular pET-32a(+) vector (1 µg, Millipore Sigma, catalog # 69015-3) was incubated with 1 µL each of restrictions enzymes NdeI (New England Biosciences, catalog # R0111S) and NotI-HF (New England Biosciences, catalog # R3189S) in cutSmart Buffer (New England Biolabs, catalog #B7204S) at 37°C for 1 hour. The entire restriction digest reaction was run on a 0.8% agarose gel at 150V for 45 minutes and linear 5.4 kbp and 0.5 kbp fragments were observed using a blue light transilluminator. The larger 5.4 kbp fragment was excised from the gel and purified using a Monarch DNA Gel Extraction Kit (NEB, catalog # T1020S). The concentration of purified, linearized pET-32(a)+ was determined by absorbance at 260 nm. Next, 33.3 ng of purified, linearized pET-32(a)+ and 100 ng of respective gBlock DNA fragment (Integrated DNA Technologies, Coralville, IA) encoding mCherry constructs were combined in a 10 µL Gibson Assembly reaction[6] containing HiFI DNA Assembly Master Mix (NEB, catalog #E2621L) and incubated at 50°C for 1 hour to generate circular pET-32(a)+ vectors containing coding sequences for mCherry constructs. Circularized plasmids from the previous step were transformed into NEB 5-alpha competent E. coli (NEB, catalog # C2987H) as follows. Frozen stocks of cells were thawed on ice for 10 minutes. Upon thawing 4 µL of the previous Gibson Assembly reaction was added to cells and incubated on ice for 30 minutes . Cells incubated with plasmid were then subjected to heat shock at 42 °C for 30 seconds and placed on ice for 5 minutes. 900 µL of SOC outgrowth medium (NEB, catalog # B9020S) was added to cells and cells were incubated at 37 °C for 1 hour with shaking at 200rpm. Agar plates containing 100

μg/mL carbenicillin were inoculated with 100 μL of transformed cells and grown overnight at 37 °C. 5 single colonies per construct were picked and inoculated into liquid cultures containing 5 mL LB + 100 μg/mL carbenicillin and grown for 16 hours at 37 °C. Pure plasmid was isolated from 5 mL cultures using Qiaprep Spin Miniprep Kit (Qiagen, catalog # 27106) and sequences were confirmed by Sanger sequencing at the UC Berkeley DNA Sequencing Facility. Plasmids containing the precise coding sequence for each construct were transformed into chemically competent BL21 E. coli (NEB, catalog # C2530H) following the same transformation protocol detailed above for large scale protein expression.

mCherryC+ gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCACCACCATCACCA
TCATATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTC
AAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGA
GGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCC
TGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAA
GCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGA
GCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGC
AGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCC
CCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGG
ACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTAC
GACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTA
CAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAG
TACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGGAAAAT
CTTTATTTTCAGGGAATGTGCGCCTACGACGGAGACTACAAAGACGACGACGACAAATAAG
CGGCCGCACTCGAGCACC

mCherryC- gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCACCACCATCACCA
TCATATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTC
AAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGA
GGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCC
TGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAA
GCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGA
GCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGC
AGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCC
CCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGG
ACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTAC
GACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTA
CAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAG
TACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGGAAAAT
CTTTATTTTCAGGGAATGGCCGCCTACGACGGAGACTACAAAGACGACGACGACAAATAAG
CGGCCGCACTCGAGCACC

mCherry137+ gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATG
CGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA
GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGT
GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCC
TACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA
AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCC
TCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCC
GACATGTGCGCCTACGACGGAGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGC
CTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGC
TGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGA
AGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACA
ACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGC
GGCATGGACGAGCTGTACAAGCACCACCATCACCATCATTAAGCGGCCGCACTCGAGCACC

mCherry174+ gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATG
CGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA
GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGT
GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCC
TACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA
AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCC
TCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCC
GACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTAC
CCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACATGTG
CGCCTACGACGGAGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAA
GCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAA
CGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCG
GCATGGACGAGCTGTACAAGCACCACCATCACCATCATTAAGCGGCCGCACTCGAGCACC

mCherry192+ gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATG
CGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA
GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGT
GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCC
TACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA
AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCC
TCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCC
GACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTAC
CCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCG

GCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGATGTGCGCCT
ACGACGGACAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACA
ACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGC
GGCATGGACGAGCTGTACAAGCACCACCATCACCATCATTAAGCGGCCGCACTCGAGCACC

mCherry211+ gBlock Sequence
TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATG
CGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA
GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGT
GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCC
TACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA
AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCC
TCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCC
GACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTAC
CCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCG
GCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCG
GCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGATGTGCGCCTACGA
CGGAGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCG
GCATGGACGAGCTGTACAAGCACCACCATCACCATCATTAAGCGGCCGCACTCGAGCACC

C. Design of Rop variants

Loop-modified substrates (RopN, RopC, RopM, RopCG4) are analogs of the Rop sequence associated with Uniprot ID: P03051. All contained an N-terminal **FLAG** purification tag, and one of five **ten-amino acid sequences** in place of the native Asp30-Ala31 sequence of Rop. They also all contained a C-terminal **6xHis** purification tag. The full sequences of the Rop translation products are provided below.

RopN
M**DYKDDDDK**MTKQEKTALNMARFIRSQTLTLLEKLNEL**MCAYDGGGGG**DEQADICESLHDHA
DELYRSCLARFGDDGENL**HHHHHH**

RopM
M**DYKDDDDK**MTKQEKTALNMARFIRSQTLTLLEKLNEL**GGMCAYDGGG**DEQADICESLHDHA
DELYRSCLARFGDDGENL**HHHHHH**

RopC
M**DYKDDDDK**MTKQEKTALNMARFIRSQTLTLLEKLNEL**GGGGMCAYDG**DEQADICESLHDHA
DELYRSCLARFGDDGENL**HHHHHH**

RopC-

MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGGGMAAYDGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

RopCG$_4$

MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGGGMCGGGGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

D. Cloning Rop constructs into pET-32(a)+ vector

The sequences encoding RopN, RopM, RopC, RopC-, and RopCG$_4$ were cloned into a pET-32a(+) plasmid as follows. Circular pET-32a(+) vector (1 µg, Millipore Sigma, catalog # 69015-3) was incubated with 1µL each of restrictions enzymes NdeI (New England Biosciences, catalog # R0111S) and NotI-HF (New England Biosciences, catalog # R3189S) in cutSmart Buffer (New England Biolabs, catalog #B7204S) at 37°C for 1 hour. The entire restriction digest reaction was run on a 0.8% agarose gel at 150V for 45 minutes and 5.4kbp and 0.5kbp fragments were observed using a blue light transilluminator. The larger 5.4kbp fragment was excised from the gel and purified using a Monarch DNA Gel Extraction Kit (NEB, catalog # T1020S). Concentration of linearized pET-32(a)+ vector was determined by absorbance at 260 nm. 33.3 ng of purified, linearized pET-32(a)+ vector and 100 ng of respective gBlock DNA fragment (Integrated DNA Technologies, Coralville, IA) encoding Rop constructs were combined in a 10µL Gibson Assembly reaction containing HiFI DNA Assembly Master Mix (NEB, catalog #E2621L) and incubated at 50°C for 1 hour to generate circular pET-32(a)+ vectors containing coding sequences for Rop constructs. Circularized plasmids from the previous step were transformed into NEB 5-alpha competent *E. coli* (NEB, catalog # C2987H). First, cells were thawed on ice for 10 minutes after which 4 µL of the previous Gibson Assembly reaction was added to cells and placed back on ice for 30 minutes . After addition of plasmid, cells were subjected to heat shock at 42°C for 30 seconds and placed on ice for 5 minutes. 900 µL of SOC outgrowth medium (NEB, catalog # B9020S) was added to cells and cells were incubated at 37 °C for 1 hour with shaking at 200rpm. Agar plates containing 100 µg/mL carbenicillin were inoculated with 100 µL of transformed cells and grown overnight at 37°C. 5 single colonies per construct were picked and inoculated into 5 mL LB + 100 µg/mL carbenicillin and grown for 16 hours at 37°C. Pure plasmid was isolated from 5 mL cultures using Qiaprep Spin Miniprep Kit (Qiagen, catalog # 27106) and sequences were confirmed by Sanger sequencing at the UC Berkeley DNA Sequencing Facility. Plasmids containing the precise coding sequence for each construct were transformed into chemically competent BL21 *E. coli* (NEB, catalog # C2530H) for large scale protein expression following the same transformation protocol detailed above.

RopN gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGC
CAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGATGTGCGCCTACGACGGAGGGGGC
GGTGGCGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCCACCACCATCACCATCATTA
AGCGGCCGCACTCGAGCACC

RopM gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGC
CAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGGCGGGATGTGCGCCTACGACGGA
GGTGGCGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCCACCACCATCACCATCATTA
AGCGGCCGCACTCGAGCACC

RopC gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGC
CAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGGCGGGGGGGGGAATGTGCGCCTAC
GACGGAGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCCACCACCATCACCATCATTA
AGCGGCCGCACTCGAGCACC

RopC- gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGC
CAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGGCGGAGGTGGAATGGCCGCCTAC
GACGGAGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCCACCACCATCACCATCATTA
AGCGGCCGCACTCGAGCACC

RopCG_4 gBlock Sequence

TCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGACTACAAAGACGA
CGACGACAAAATGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGC
CAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGGCGGGGGTGGAATGTGCGGTGGG
GGAGGAGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCCACCACCATCACCATCATTA
AGCGGCCGCACTCGAGCACC

E. Expression and Purification of mCherry variants

Starter cultures of 2 mL of Miller's LB Broth (AmericanBio, catalog # AB01201) supplemented with 100 µg/mL carbenicillin were inoculated with a single colony of *E. coli* BL21 (DE3) harboring the plasmid of interest and grown overnight at 37°C with shaking at 200 rpm.The starter culture (2 mL) was used to inoculate a 200 mL expression culture of Miller's LB Broth also supplemented with 100 µg/mL carbenicillin. The expression culture was grown at 37°C with shaking at 200 rpm to an OD_{600} of 0.6 at which point it was induced with 1 mM IPTG, transferred

to a 20ºC incubator, and grown for 24 hours with shaking at 200 rpm. The expression culture was harvested by centrifugation at 4,300xg at 4ºC for 45 minutes. The resulting cell pellet was suspended in 10 mL of Lysis Buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) containing 1 tablet of cOmplete, mini EDTA-free ULTRA protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell suspension was disrupted by sonication on ice (Branson Sonifier 250, 3 cycles of 30 second pulse at 30% duty cycle and microtip limit of 5 followed by 60 second pause). The cell lysate was cleared by centrifugation at 23,000xg at 4ºC for 20 minutes. TALON® Metal Affinity Resin (2 mL) (Takara Biosciences, catalog # 635504) was equilibrated with Lysis Buffer, added to the cleared cell lysate, and incubated on a rotisserie at 4ºC for 1 hour. The TALON® resin-lysate mixture was then passed through a gravity flow Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA). Non-specifically bound proteins were removed by washing the Ni-NTA column with 10 mL of Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole pH 8.0). The 6xHis-tagged protein was eluted by washing the Ni-NTA column with 5 mL of Elution Buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole pH 8.0). The purified protein was loaded into a 3-12 mL Slide-A-Lyzer Dialysis Cassette with a 10 kDa molecular weight cut off (Thermo Scientific, Waltham, MA) and dialyzed against 1 L of Storage Buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) at 4ºC for at least 18 hours. The dialyzed protein was concentrated using Amicon Ultra-15 mL centrifugal filters with a 10 kDa molecular weight cut off (MilliporeSigma, Burlington, MA) according to the manufacturer's instructions. The purified protein was quantified using absorbance at 280 nm, diluted to 1 mM using Storage Buffer, snap frozen as single-use aliquots, and stored at -80ºC. The typical expression yield of mCherry constructs using the above protocol was 90 mg/L of *E. coli* culture.

F. Expression and Purification of Rop Constructs

Starter cultures of 2 mL of Miller's LB Broth (AmericanBio, catalog # AB01201) supplemented with 100 µg/mL carbenicillin were inoculated with a single colony of *E. coli* BL21 (DE3) harboring the plasmid of interest and grown overnight at 37ºC with shaking at 200 rpm.The starter culture (2 mL) was used to inoculate a 200 mL expression culture of Miller's LB Broth also supplemented with 100 µg/mL carbenicillin. The expression culture was grown at 37ºC with shaking at 200 rpm to an OD_{600} of 0.6 at which point it was induced with 1 mM IPTG, transferred to a 20ºC incubator, and grown for 24 hours with shaking at 200 rpm. The expression culture was harvested by centrifugation at 4,300xg at 4ºC for 45 minutes. The resulting cell pellet was suspended in 10 mL of Lysis Buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) containing 1 tablet of cOmplete, mini EDTA-free ULTRA protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell suspension was disrupted by sonication on ice (Branson Sonifier 250, 3 cycles of 30 second pulse at 30% duty cycle and microtip limit of 5 followed by 60 second pause).The cell lysate was cleared by centrifugation at 23,000xg at 4ºC for 20 minutes. TALON® Metal Affinity Resin (2 mL) (Takara Biosciences, catalog # 635504) was equilibrated with lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0). The equilibrated Ni-NTA agarose (2 mL) was added to the cleared cell lysate and incubated on a rotisserie at 4ºC for 1 hours. The TALON® resin-lysate mixture was then passed through a gravity flow Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA). Non-specifically bound proteins were removed by washing the

TALON® column with 10 mL of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole pH 8.0). The 6xHis-tagged protein was eluted by washing the TALON® column with 5 mL of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole pH 8.0). The purified protein was loaded into a 3-12 mL Slide-A-Lyzer Dialysis Cassette with a 3.5 kDa molecular weight cut off (Thermo Scientific, Waltham, MA) and dialyzed against 1 L of storage buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) at 4ºC for at least 18 hours. The dialyzed protein was concentrated using Amicon Ultra-15 mL centrifugal filters with a 3 kDa molecular weight cut off (MilliporeSigma, Burlington, MA) according to the manufacturer's instructions. The purified protein was quantified using absorbance at 280 nm, diluted to 1 mM using storage buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4), snap frozen as single use aliquots, and stored at -80ºC. The typical expression yield of Rop construct using the above protocol was 40 mg/L of *E. coli* culture.

G. Characterization of mCherry and Rop Constructs

SDS-PAGE. Purified proteins (4 µg) were mixed (4:1) with SDS-PAGE sample buffer (5% β-Mercaptoethanol, 0.02% bromophenol blue, 30% glycerol, 10% SDS, 250 mM Tris-HCl, pH 6.8) and incubated at 95ºC for 5 minutes before being applied to a 4-15% mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Hercules, CA) run at 200 V for 30 minutes in Tris-Glycine-SDS running buffer (3 g/L tris, 14.4 g/L glycine, 1 g/L sodium dodecyl sulfate, pH 8.3) alongside a lane containing Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Hercules, CA). Protein bands were visualized using Coomassie stain (1 g/L Coomassie Brilliant Blue in methanol:water:acetic acid (5:4:1)) and imaged using a Bio-Rad Chemidoc MP Imaging System. Band intensities were quantified using the gel analysis tool of FIJI.[5]

H. LC-MS analysis of purified proteins

LC-MS analysis was performed on an Agilent 1290 Infinity II HPLC connected to an Agilent 6530B QTOF AJS-ESI. The mobile phase for LC-MS was water and acetonitrile with 0.1% (v/v) formic acid and a flow rate of 0.4 mL/min. Each sample was injected onto a Poroshell 300SB-C8 column (2.1 x 75 mm, 5-Micron, room temperature, Agilent) using a linear gradient from 5 to 95% acetonitrile over 9 minutes after an initial hold at 5% acetonitrile for 0.5 minutes (0.4 mL/min). The following parameters were used during acquisition: Fragmentor voltage 225 V, gas temperature 300ºC, gas flow 10 L/min, sheath gas temperature 350ºC, sheath gas flow 11 L/min, nebulizer pressure 35 psi, skimmer voltage 65 V, Vcap 5000 V, 1 spectra/s. Intact protein masses were obtained via deconvolution using the Maximum Entropy algorithm in Mass Hunter Bioconfirm (V10, Agilent).

V. Reactions with MicD-F and/or ArtGox

A. Reaction of peptides and proteins with MicD-F

A typical reaction scale for analysis via LC-MS was 30 µL. Peptide substrate stock (3 µL) suspended at 1 mM (10 mM bicine, 150 mM NaCl, 1 mM TCEP, pH 9.0) was added to 24 µL of reaction buffer (6.25 mM ATP, 6.25 mM $MgCl_2$, 100 mM bicine, 150 mM NaCl, 1 mM TCEP, pH 8.0 or 9.0). Separately, an aliquot of MicD-F (500 µM in 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) was thawed on ice. The thawed enzyme aliquot was then diluted with cold storage buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) to a concentration equal to 10x the desired enzyme concentration. The peptide and enzyme solutions were then incubated at 37ºC for 15 minutes. After temperature equilibration, enzyme solution (3 µL) was added to the peptide solution and gently pipette mixed. The resulting solution was then incubated at 37ºC for the indicated amount of time before LC-MS analysis.

Reactions of proteins with MicD-F were performed in the manner described above with minor modifications. Namely, the substrate stock was a 1 mM solution of protein in 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4.

B. Reaction of peptides and proteins with MicD-F and ArtGox in tandem

A typical reaction scale for analysis via LC-MS was 30 µL. Peptide substrate stock (3 µL) suspended at 1 mM in 10 mM bicine, 150 mM NaCl, 1 mM TCEP, pH 9.0 was added to 21 µL of reaction buffer (7.14 mM ATP, 7.14 mM $MgCl_2$, 2.86 mM FMN, 100 mM bicine, 150 mM NaCl, 1 mM TCEP, pH 8.0 or 9.0). Separately, aliquots of MicD-F (500 µM in 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) and ArtGox (800 µM in 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) were thawed on ice. The thawed enzyme aliquots were then diluted with cold storage buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4) to a concentration equal to 10x the desired enzyme concentration. The peptide and enzyme solutions were then incubated at 37ºC for 15 minutes. After temperature equilibration, enzyme solutions (3 µL of each) were added to the peptide solution and gently pipette mixed. The resulting solution was then incubated at 37ºC for the indicated amount of time before LC-MS analysis.

Reactions of proteins with MicD-F and ArtGox were performed in the manner described above with minor modifications. The substrate stock was a 1 mM solution of protein in 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.4. Reactions were carried out at either 25ºC or 37ºC as indicated.

C. LC-MS analysis of reactions with peptide substrates

To remove 6xHis-tagged enzymes, Ni-NTA (Qiagen, Hilden, Germany) slurry and crude reaction were mixed 1:1 by volume and incubated on ice for 30 minutes with occasional agitation. The Ni-NTA resin was then removed by centrifugation at 21,300 x g for 10 minutes at 4ºC. Enzyme-depleted reaction mixture (1 µL) was used for LC-MS analysis which was performed on an Agilent 1290 Infinity II HPLC connected to an Agilent 6530B QTOF AJS-ESI. The mobile phase for LC-MS was water and acetonitrile with 0.1% (v/v) formic acid and a flow rate of 0.7 mL/min. Each sample was injected onto an Eclipse XDB C-18 column (2.1 x 50 mm, 1.8-Micron,

room temperature, Agilent) and separated using a linear gradient from 5 to 95% acetonitrile over 4.5 minutes after an initial hold at 5% acetonitrile for 0.5 minutes. The following parameters were used during acquisition: Fragmentor voltage 175 V, gas temperature 300ºC, gas flow 8 L/min, sheath gas temperature 350ºC, sheath gas flow 11 L/min, nebulizer pressure 35 psi, skimmer voltage 65 V, Vcap 3500 V, 1 spectra/s.

D. LC-MS analysis of reactions with protein substrates

Unprocessed reaction mixture (1 µL) was used for LC-MS analysis which was performed on an Agilent 1290 Infinity II HPLC connected to an Agilent 6530B QTOF AJS-ESI. The mobile phase for LC-MS was water and acetonitrile with 0.1% (v/v) formic acid and a flow rate of 0.4 mL/min. Each sample was injected onto a Poroshell 300SB-C8 column (2.1 x 75 mm, 5-Micron, room temperature, Agilent) using a linear gradient from 5 to 55% acetonitrile over 8 minutes after an initial hold at 5% acetonitrile for 2 minutes (0.4 mL/min). The following parameters were used during acquisition: Fragmentor voltage 225 V, gas temperature 300ºC, gas flow 10 L/min, sheath gas temperature 350ºC, sheath gas flow 11 L/min, nebulizer pressure 35 psi, skimmer voltage 65 V, Vcap 5000 V, 1 spectra/s. Intact protein masses were obtained via deconvolution using the Maximum Entropy algorithm in Mass Hunter Bioconfirm (V10, Agilent).

VI. Characterization of Rop Variants

A. Purification of RopC-U and RopC-Z via size-exclusion chromatography

Reactions to synthesize and purify thiazoline (RopC-U)- and thiazole (RopC-Z)-modified Rop variants were carried out in a total reaction volume of 1.5 mL. All stock solutions and reaction components were scaled up in accordance with the analytical scale reaction protocols described above. To synthesize RopC-U, RopC (100 µM) was reacted with MicD-F (50 µM) at pH 9.0 and 37ºC. To synthesize RopC-Z, RopC (100 µM) was reacted with MicD-F (50 µM) and ArtGox (80 µM) at pH 9.0 and 37ºC. Reaction progress was monitored in the manner described above for LC-MS analysis of analytical scale protein reactions. Once the reaction was complete as judged by LC-MS, RopC variants were separated from the crude reaction mixture via size exclusion chromatography (SEC).

A HiLoad® 16/600 Superdex® 75 pg column (stored and operated at 4℃) was washed with 2 column volumes (CV) of degassed and sterile filtered MilliQ water. The column was then equilibrated in running buffer (10 mM phosphate, 100 mM NaCl, 150 µM TCEP, pH 7.0) for 2 CV. The crude reaction mixture (1.5 mL) was applied to a 5 mL sample loop. The sample loop was washed with 10 mL of running buffer at 1 mL/min to load the sample onto the column. The sample was then eluted from the column by flowing running buffer at 1 mL/min for 1.5 CV.

Fractions were collected in 1 mL aliquots for the entirety of sample application and elution. Fractions were analyzed via SDS-PAGE analysis and those containing protein of the correct molecular weight (approximately 10 kDa) were pooled and concentrated to 20 μM using Amicon Ultra-0.5 mL centrifugal filters with a 3 kDa molecular weight cut off (MilliporeSigma, Burlington, MA) according to the manufacturer's instructions. The 20 μM protein solution was equally divided into 3 parts and flash frozen on liquid nitrogen before analysis via circular dichroism.

B. Characterization of Rop variants via protease digest

To identify the site of enzymatic modification, SEC-purified samples of RopC, RopC-U, and RopC-Z (25 μL of 20 μM protein in 10 mM phosphate, 100 mM NaCl, 150 μM TCEP, pH 7.0) were treated with V8 protease (1.25 μL at 0.02 mg/mL in 10 mM phosphate, 100 mM NaCl, pH 7.0). The protease digest reactions were allowed to proceed for 16 hours at 37℃. The digested samples were then centrifuged at 20,000xg for 10 minutes at 4℃ to remove insoluble material.

The clarified reactions (10 μL) were used for LC-MS analysis which was performed on an Agilent 1290 Infinity II HPLC connected to an Agilent 6530B QTOF AJS-ESI. The mobile phase for LC-MS was water and acetonitrile with 0.1% (v/v) formic acid and a flow rate of 0.4 mL/min. Each sample was injected onto a Poroshell 300SB-C8 column (2.1 x 75 mm, 5-Micron, room temperature, Agilent) using a linear gradient from 5 to 55% acetonitrile over 8 minutes after an initial hold at 5% acetonitrile for 2 minutes (0.4 mL/min). The following parameters were used during acquisition: Fragmentor voltage 225 V, gas temperature 300ºC, gas flow 10 L/min, sheath gas temperature 350ºC, sheath gas flow 11 L/min, nebulizer pressure 35 psi, skimmer voltage 65 V, Vcap 5000 V, 1 spectra/s.

C. Characterization of Rop variants via analytical size-exclusion chromatography

To assess the homogeneity of isolated Rop variants, a solution of each protein was prepared (250 μg at 50 μM) in running buffer (10 mM phosphate, 100 mM NaCl, 150 μM TCEP, pH 7.0). A Superdex® 75 Increase 10/300 GL column (stored and operated at 4℃) was washed with 2 column volumes (CV) of degassed and sterile filtered MilliQ water. The column was then equilibrated in running buffer for 2 CV. Each sample (500 μL) was applied to a 500 μL sample loop. The sample loop was washed with 2 mL of running buffer at 0.8 mL/min to load the sample onto the column. The sample was then eluted from the column by flowing running buffer at 0.8 mL/min for 1.30 CV. To assess column performance, a gel filtration standard (Bio-Rad Laboratories, Hercules, CA, catalog number 151-1901) containing 670 kDa, 158 kDa, 44 kDa, 17 kDa, and 1.35 kDa standards was used according to the manufacturer's instructions.

D. Characterization of Rop variants via circular dichroism (CD)

RopN, RopM, and RopC were exchanged into CD buffer (10 mM phosphate, 100 mM NaCl, 150 μM TCEP, pH 7.0) using Amicon Ultra-0.5 mL centrifugal filters (MilliporeSigma, Burlington, MA) with a 3 kDa molecular weight cut-off according to the manufacturer's instructions and then

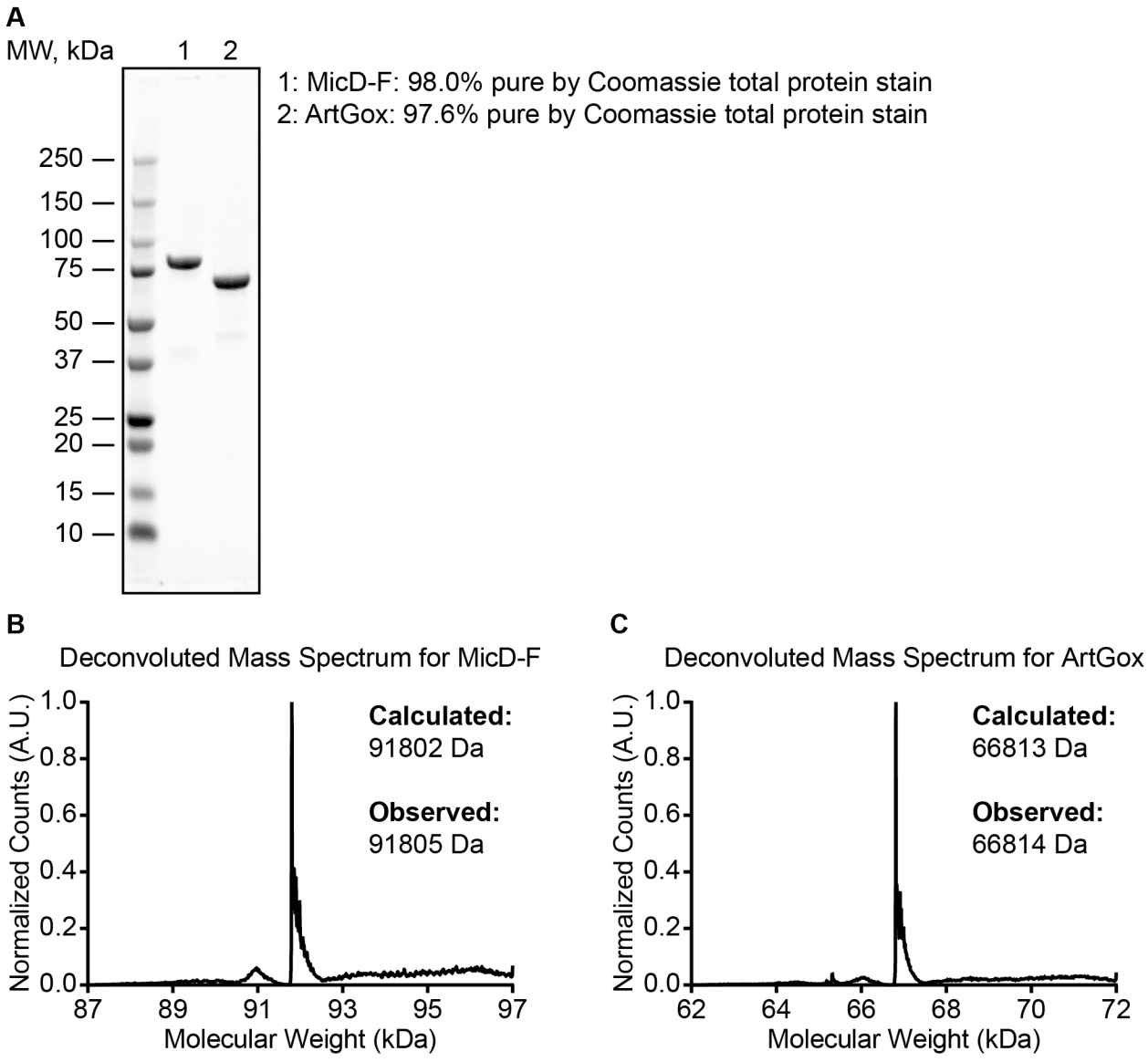
diluted with the same buffer to a concentration of 20 μM. For CD analysis each Rop variant was transferred to a 1 mm quartz cuvette. Wavelength and temperature dependent CD spectra were collected with an AVIV Biomedical, Inc. (Lakewood, NJ) Circular Dichroism Spectrometer Model 410. For wavelength-dependent spectra, initial scans were performed from 200 to 300 nm at 25°C in 2 nm increments with an averaging time of 5 seconds. For temperature melt experiments, the signal was monitored at 222 nm with an averaging time of 5 seconds. Temperature melts were performed from 5 to 90°C in 1°C increments with equilibration for 2 minutes before each measurement. Following the temperature melt the sample was returned to 25°C and the wavelength-dependent CD spectra was measured once more to assess the reversibility of the melt. Raw data (mdeg) were converted to molar ellipticity ([Θ], in $deg*cm^{2}*dmol^{-1}$) by

$$[\Theta] = (mdeg * M)/(10 * L * C) \qquad \text{Equation 1}$$

where M is the mean residual weight (116.00 g/mol for RopN, RopM, and RopC), L is the pathlength of the cuvette in centimeters, and C is the concentration of the sample in g/L.[7] The melting temperature was determined by fitting the molar ellipticity as a function of temperature to a Boltzmann sigmoidal curve using GraphPad Prism (Version 7.04).

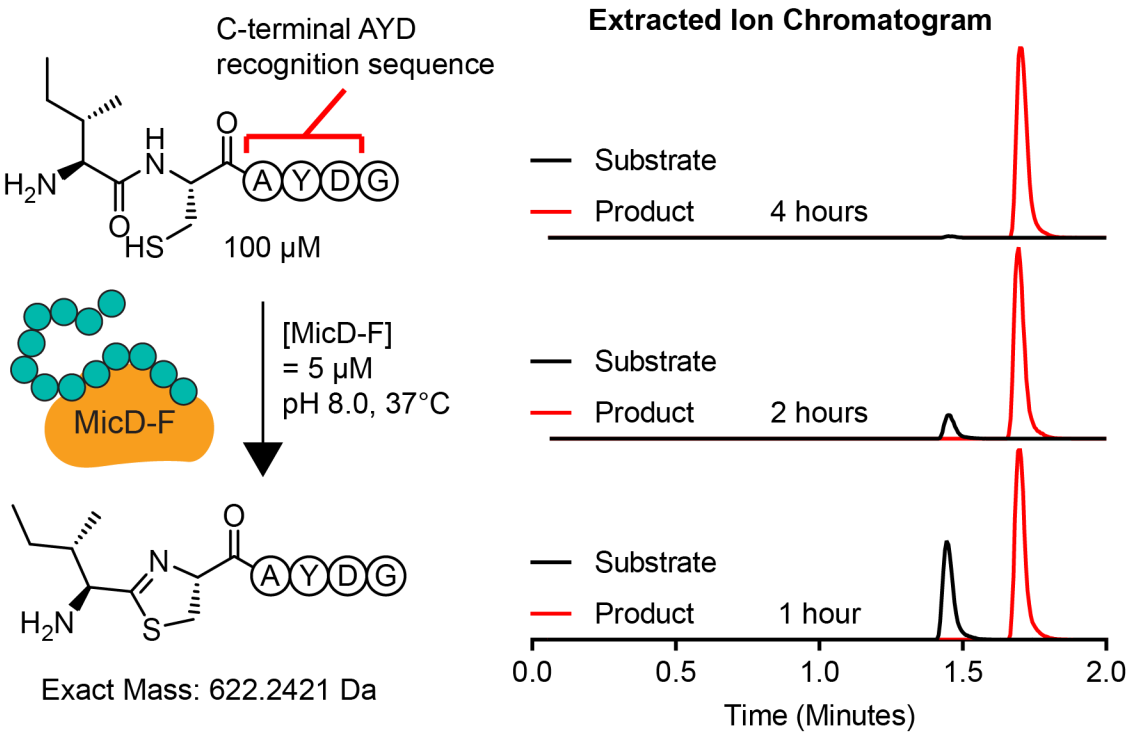
CD analysis of RopC-U and RopC-Z was performed as described for RopN, RopM, and RopC with the following modifications. Temperature melts were performed from 5 to 90°C in 2.5°C increments. Before measuring the temperature melt for RopC-Z, the protein was refolded by performing an initial melt from 5 to 90°C in 2.5°C increments as described.

VII. Supplementary Figures

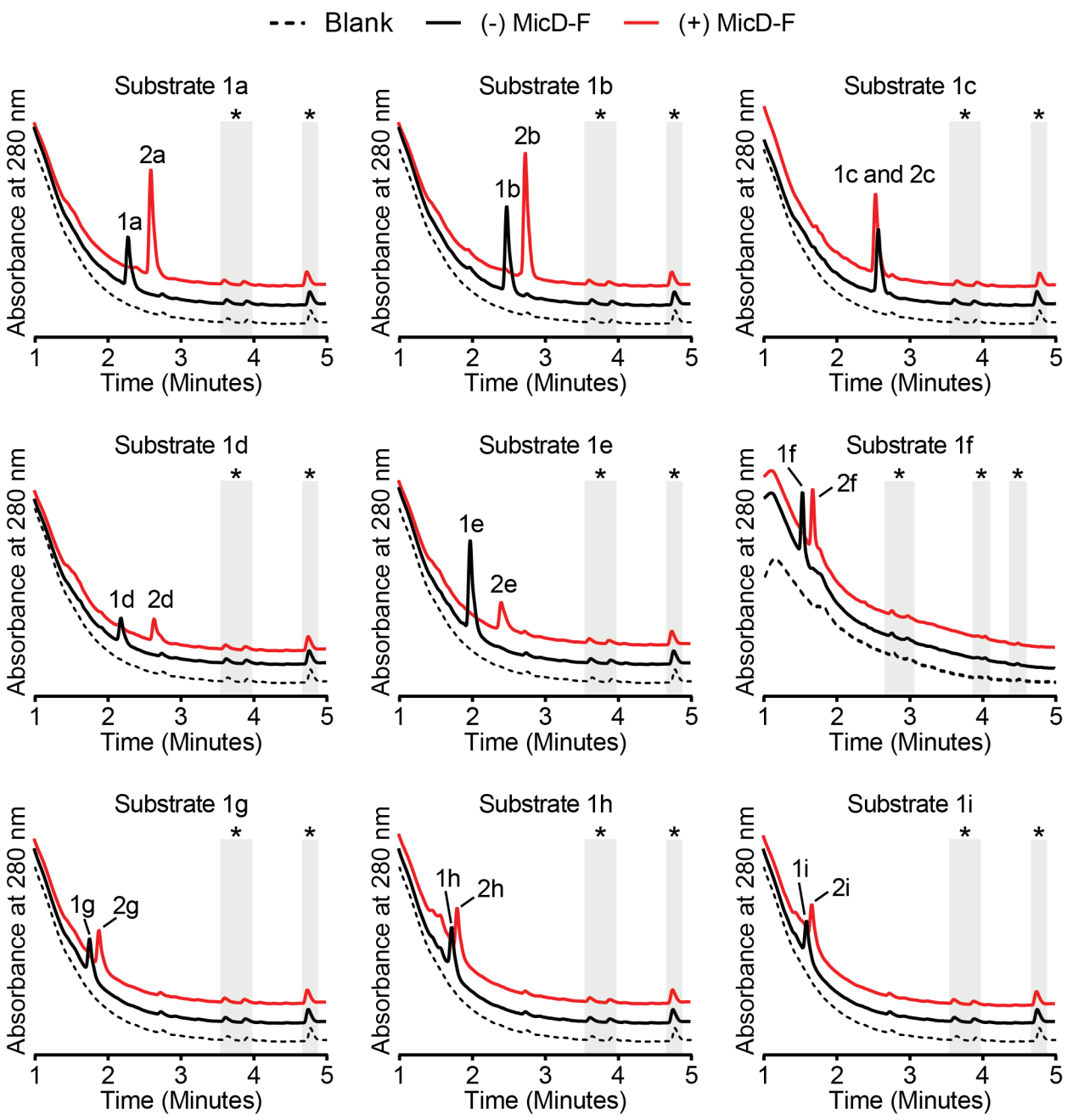


Supplementary Figure 1. Characterization of MicD-F and ArtGox purified via Ni-NTA affinity chromatography. (A) SDS-PAGE analysis of 4 µg of protein separated using a 4-15% mini-PROTEAN TGX gel run at 120 V for 60 minutes. Total protein content was visualized via Coomassie Blue staining and band intensities were quantified using the gel analysis tool of

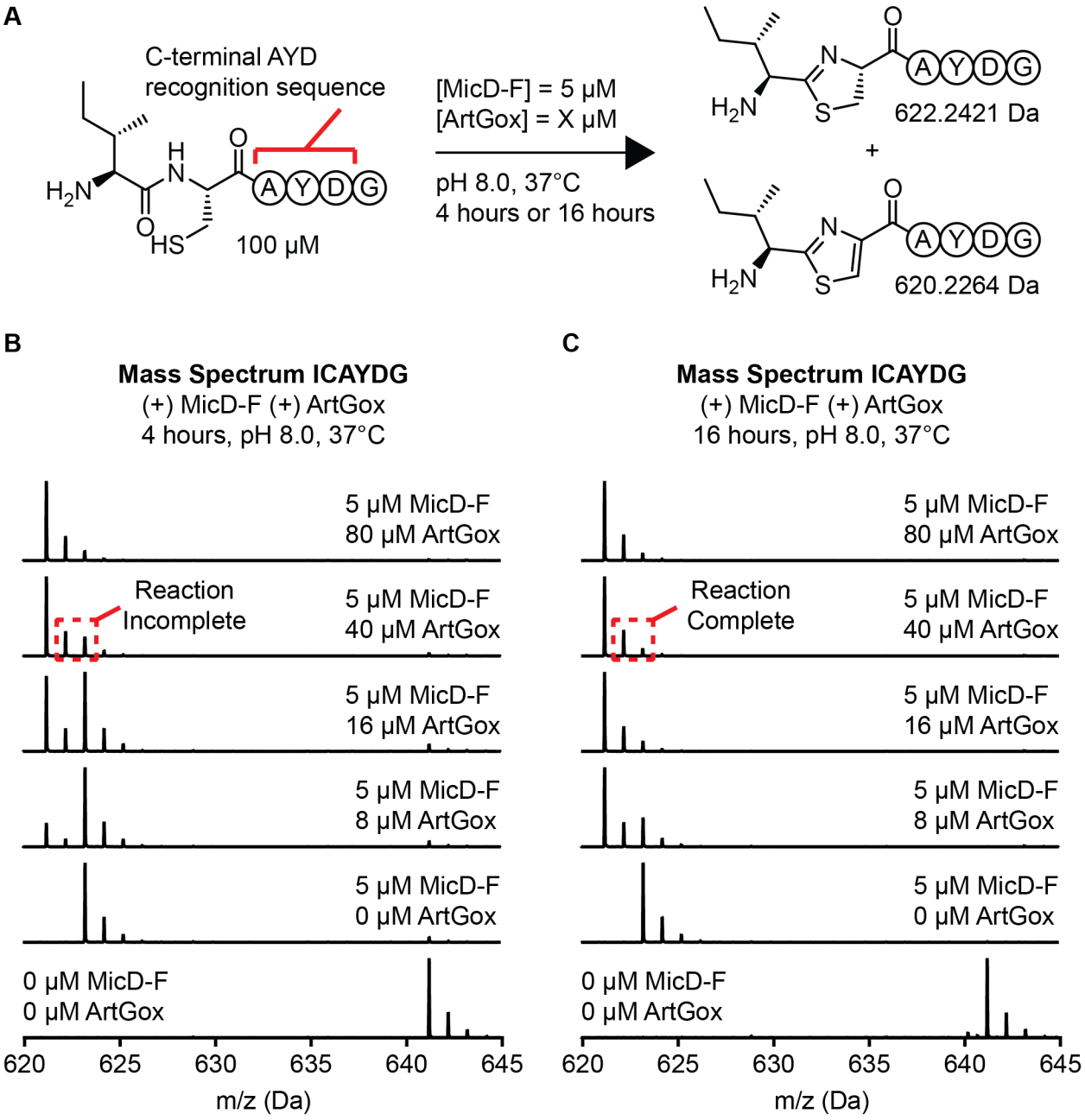
FIJI.[5] (B) Deconvoluted mass spectrum of MicD-F sample. (C) Deconvoluted mass spectrum of ArtGox sample. As expected, the N-terminal methionine residue was found to be excised from both enzymes during expression in *E. coli*.[8,9]



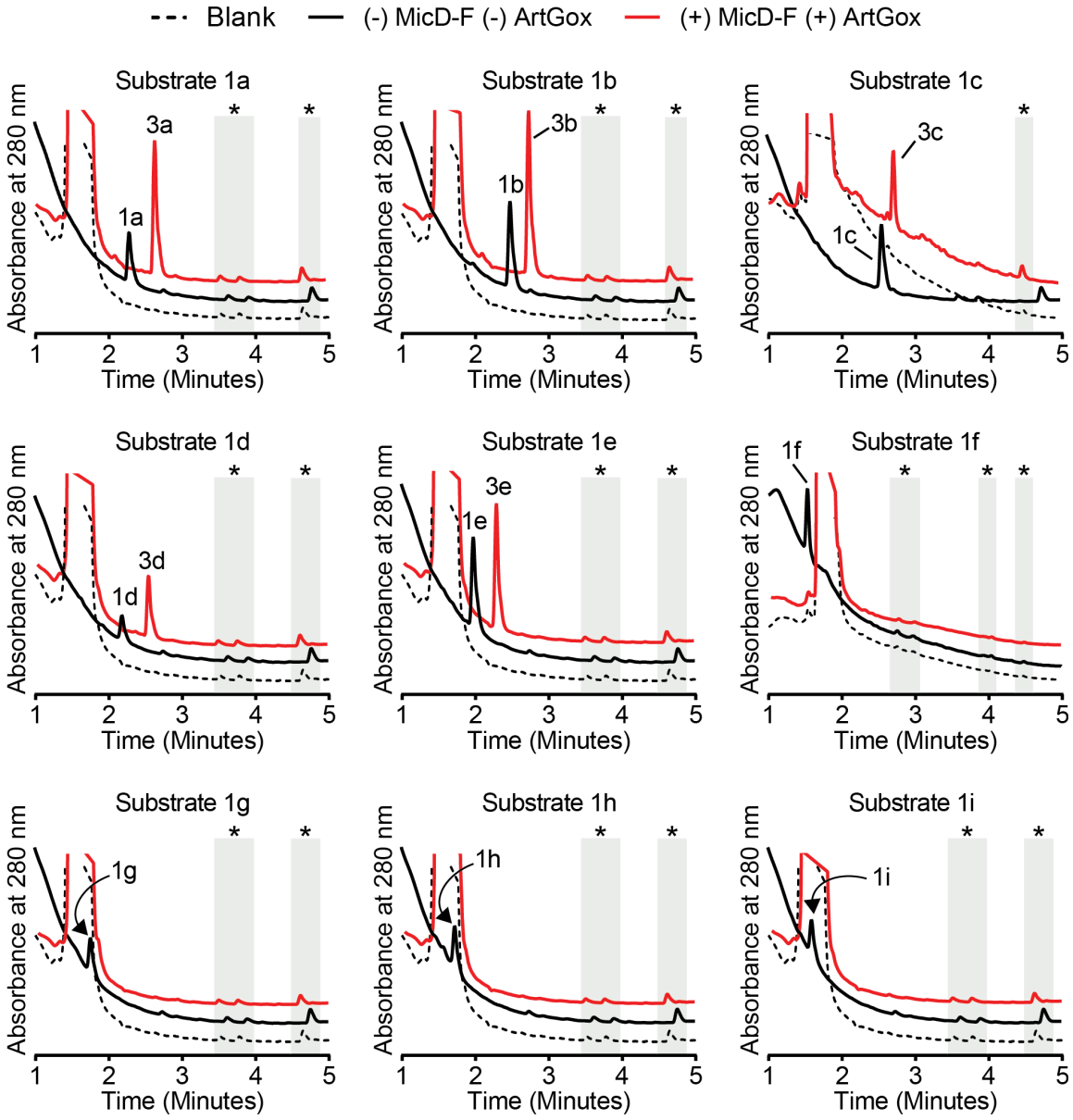
Supplementary Figure 2. Effect of time on the extent of MicD-F-promoted cyclodehydration of the model peptide ICAYDG. Examination of the extracted ion chromatograms at 1, 2, and 4 h indicated that reaction of ICAYDG with 5 mol% MicD-F was complete after 4 hours at pH 8.0 and 37°C.



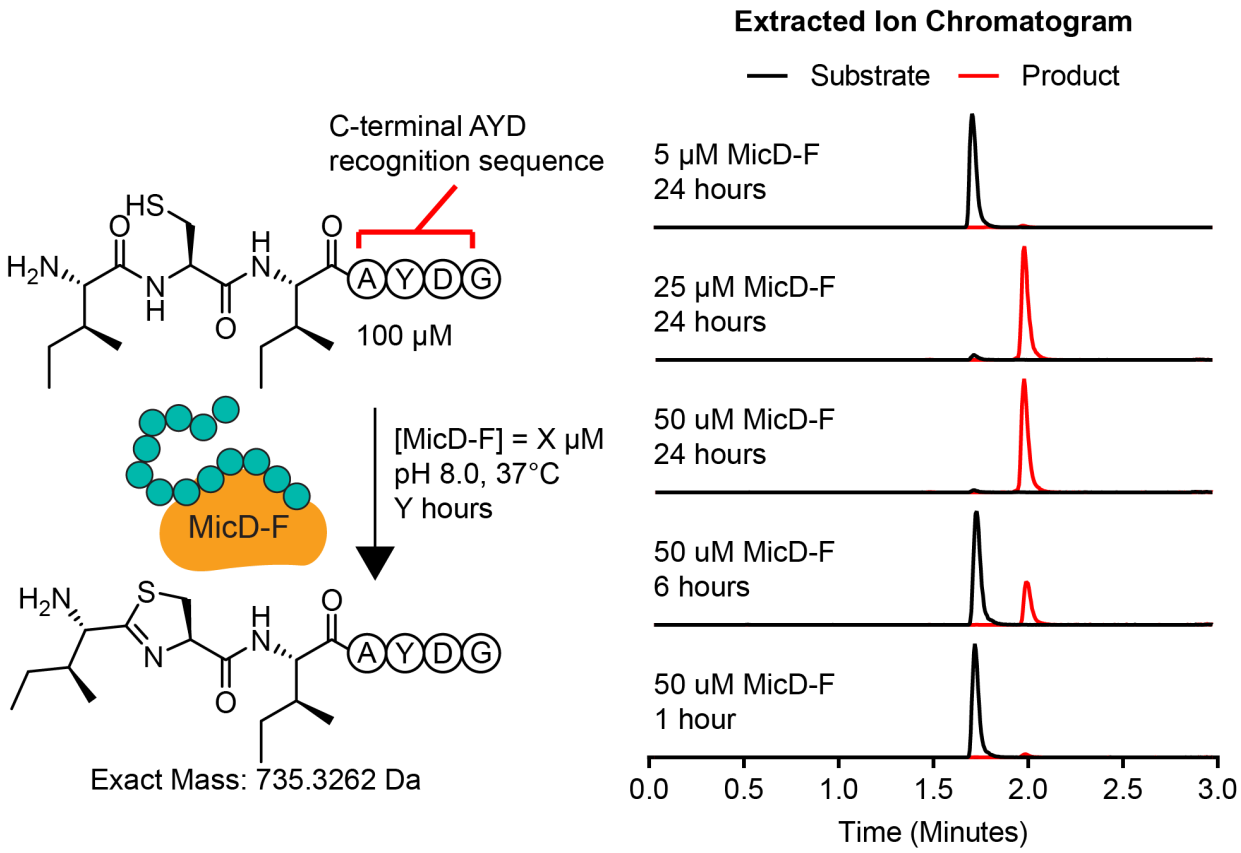
Supplementary Figure 3. UHPLC analysis of MicD-F-catalyzed cyclodehydration of substrates 1a-i. Thiazolines 2a-i are the sole reaction products when substrates 1a-i are treated with 5 mol% MicD-F at pH 8.0 and 37°C for 4 hours. Each panel shows the UHPLC trace (detection at 280 nm) of the indicated reaction mixture at the 4 hour time point for reactions that contained or lacked MicD-F. Contaminating signals identified from the absorbance baseline (dashed absorbance trace) are highlighted in grey and marked with an asterisk.



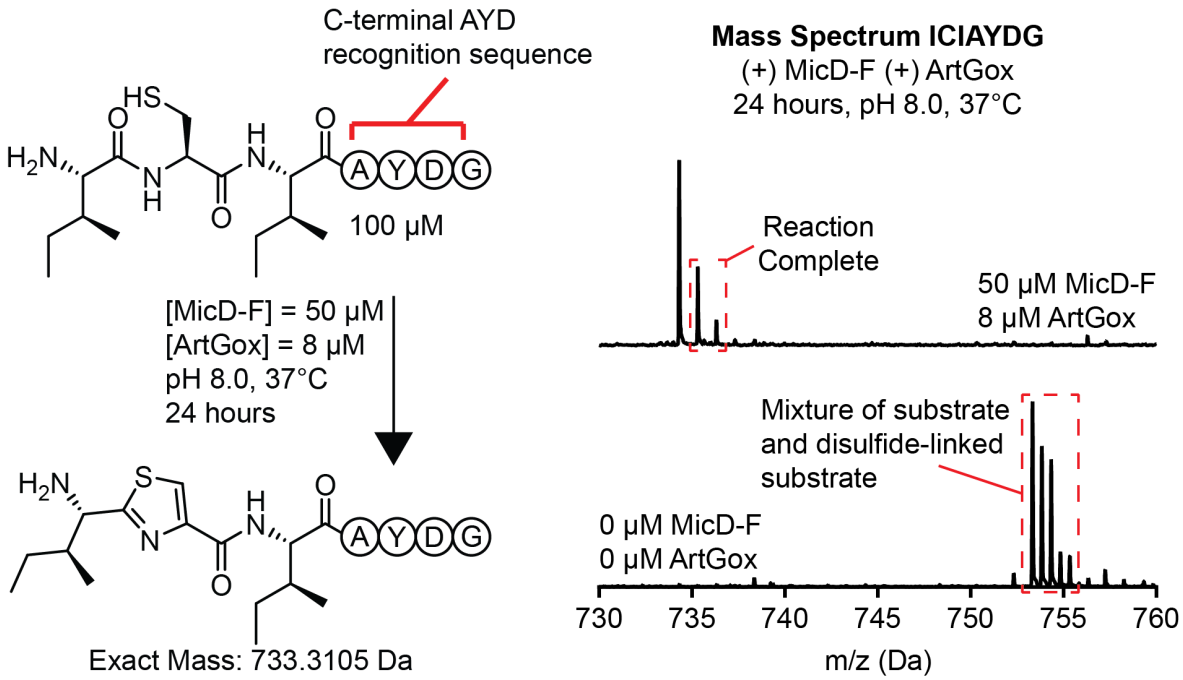
Supplementary Figure 4. Optimization of conditions for one-pot cyclodehydration and dehydrogenation of the model peptide ICAYDG by MicD-F and ArtGox. (A) Reaction of the substrate ICAYDG with 5 mol% MicD-F and 0 - 80 mol% ArtGox at pH 8.0 and 37°C. Mass spectra of reaction mixtures containing the indicated concentration of ArtGox at (B) 4 hours and (C) 16 hours show that 40 mol% ArtGox and 16 hours of reaction are required to yield full conversion to the thiazole product. The ratio between the +1 amu isotope and the +2 amu isotope (boxed, red) was monitored to confirm complete conversion to the desired thiazole product.



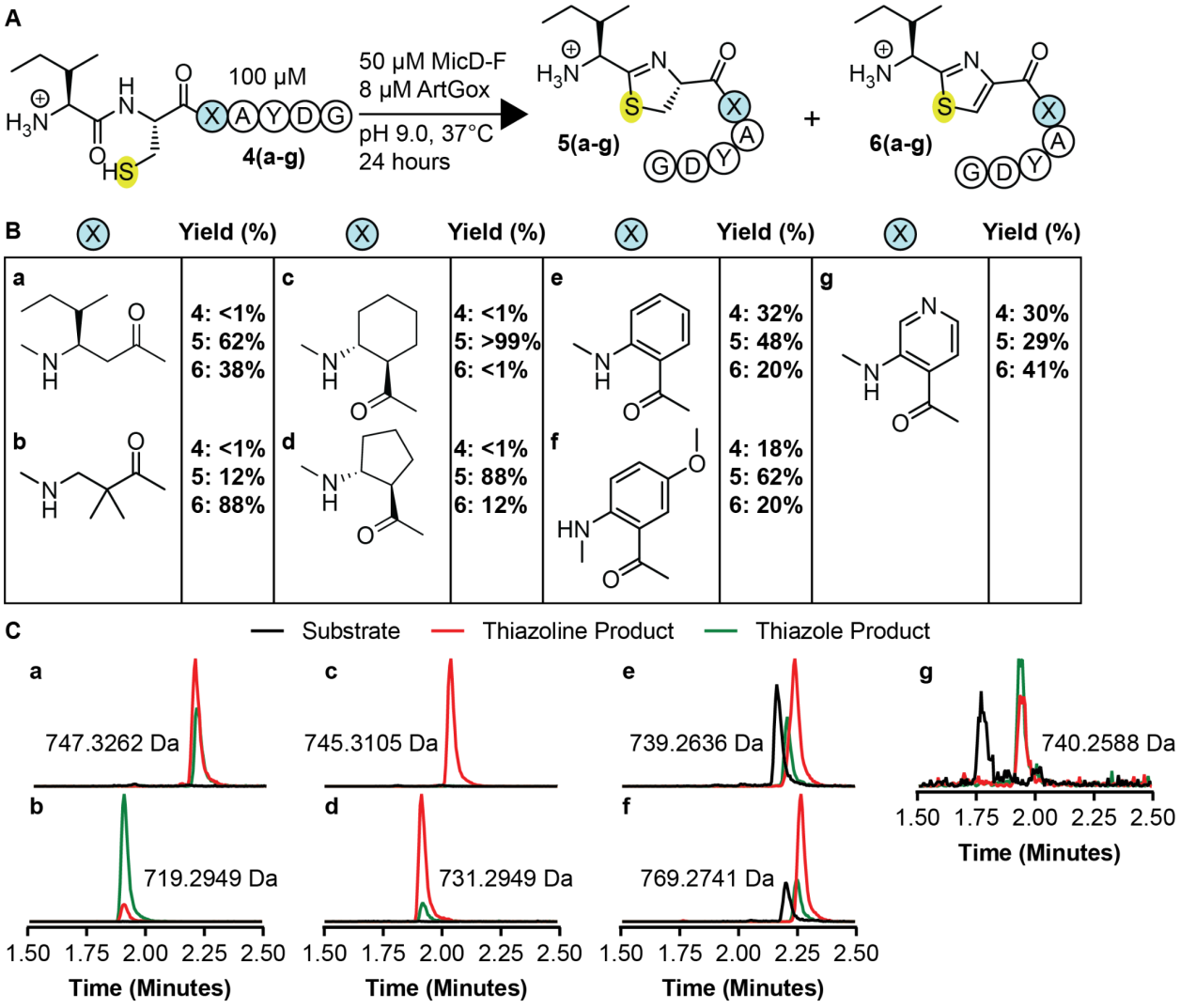
Supplementary Figure 5. UHPLC analysis of one-pot cyclodehydration and dehydrogenation of substrates 1a-i by MicD-F and ArtGox. Thiazoles 3a-e are the sole reaction products when substrates 1a-e are treated with 5 mol% MicD-F and 40 mol% ArtGox at pH 8.0 and 37°C for 16 hours. Excess flavin mononucleotide coelutes with substrates 1f-i. Therefore, UHPLC analysis cannot determine the extent of reaction for these substrates. Each panel shows the UHPLC trace (detection at 280 nm) of the indicated reaction mixture at the 16 hour time point for reactions that contained MicD-F and ArtGox (red absorbance trace). Retention time of the parent peptide as given by **Supplementary Figure 3** is provided for reference (black absorbance trace). Contaminating signals identified from the absorbance baseline (dashed absorbance trace) are highlighted in grey and marked with an asterisk.



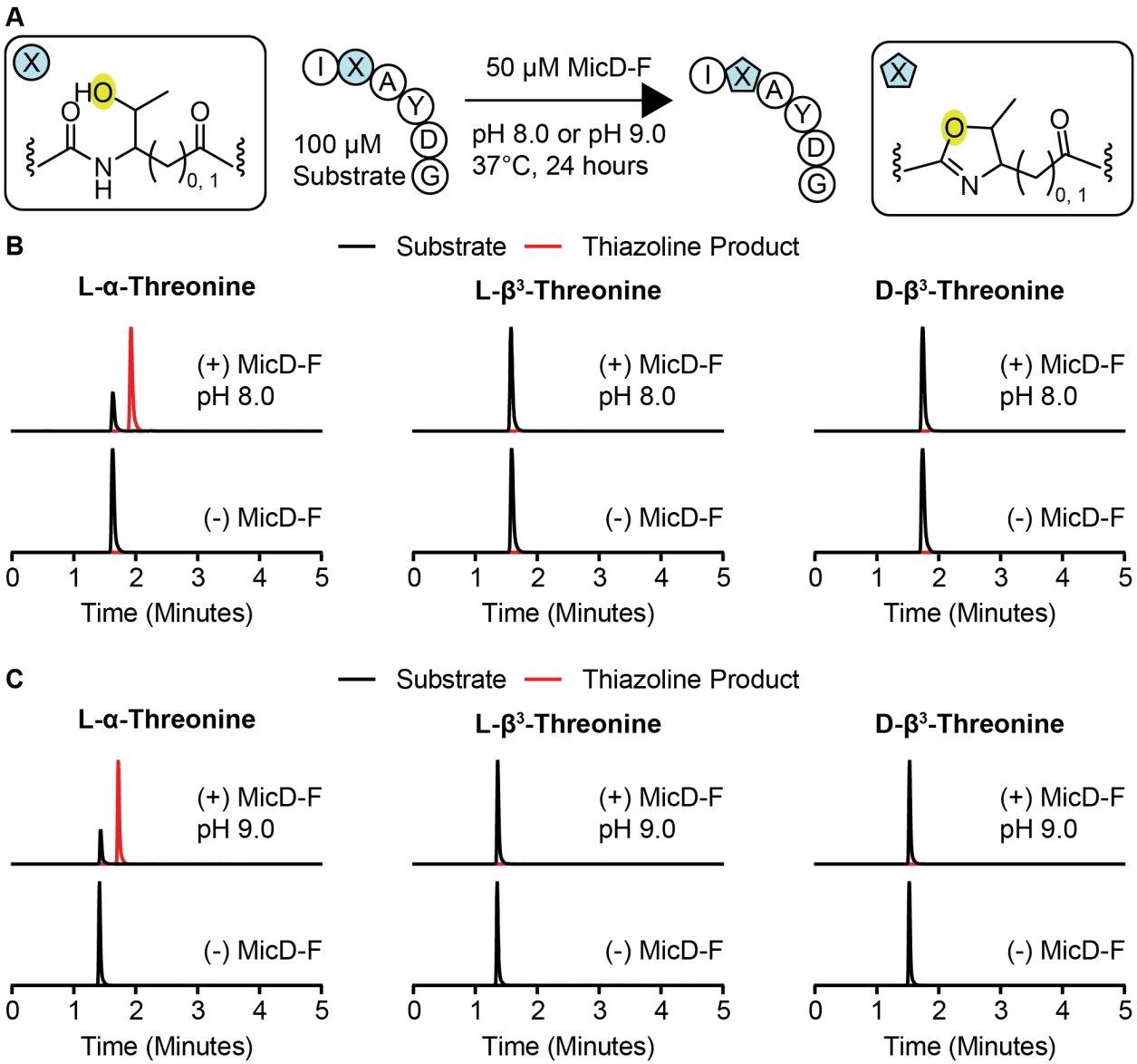
Supplementary Figure 6. Optimizing reaction conditions for the MicD-F-catalyzed cyclodehydration of the model peptide ICIAYDG. Extracted ion monitoring indicated that treatment of ICIAYDG with 50 mol% MicD-F for 24 hours at pH 8.0 and 37°C led to complete reaction.



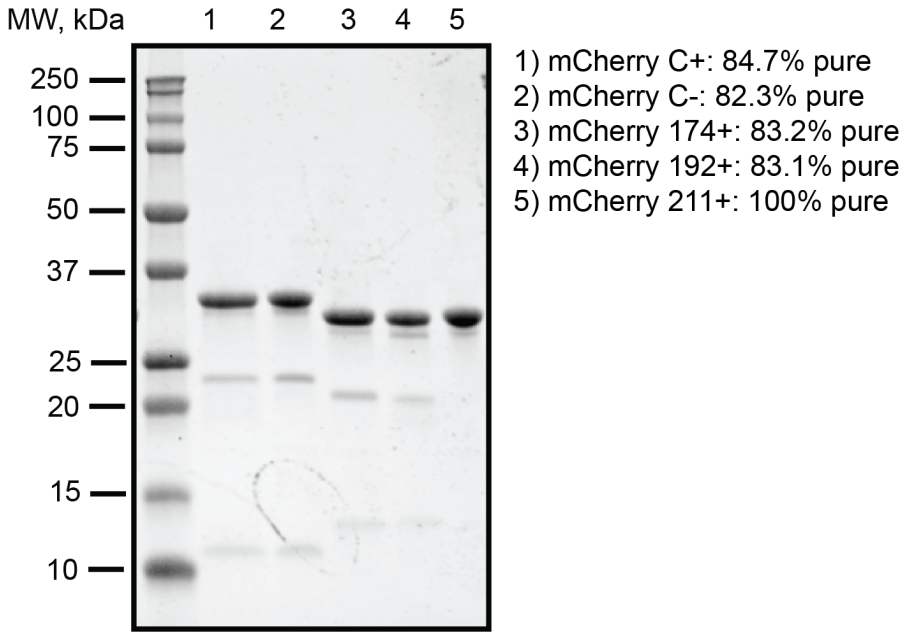
Supplementary Figure 7. Optimizing reaction conditions for one-pot cyclodehydration and dehydrogenation of the model peptide ICIAYDG in the presence of MicD-F and ArtGox. The ratio between the +1 amu isotope and the +2 amu isotope (boxed, red) was monitored to confirm complete conversion to the desired thiazole product. The mass spectral data indicates that reaction with 50 mol% MicD-F and 8 mol% ArtGox for 24 hours at pH 8.0 and 37°C was sufficient for complete conversion to the thiazole-modified peptide.



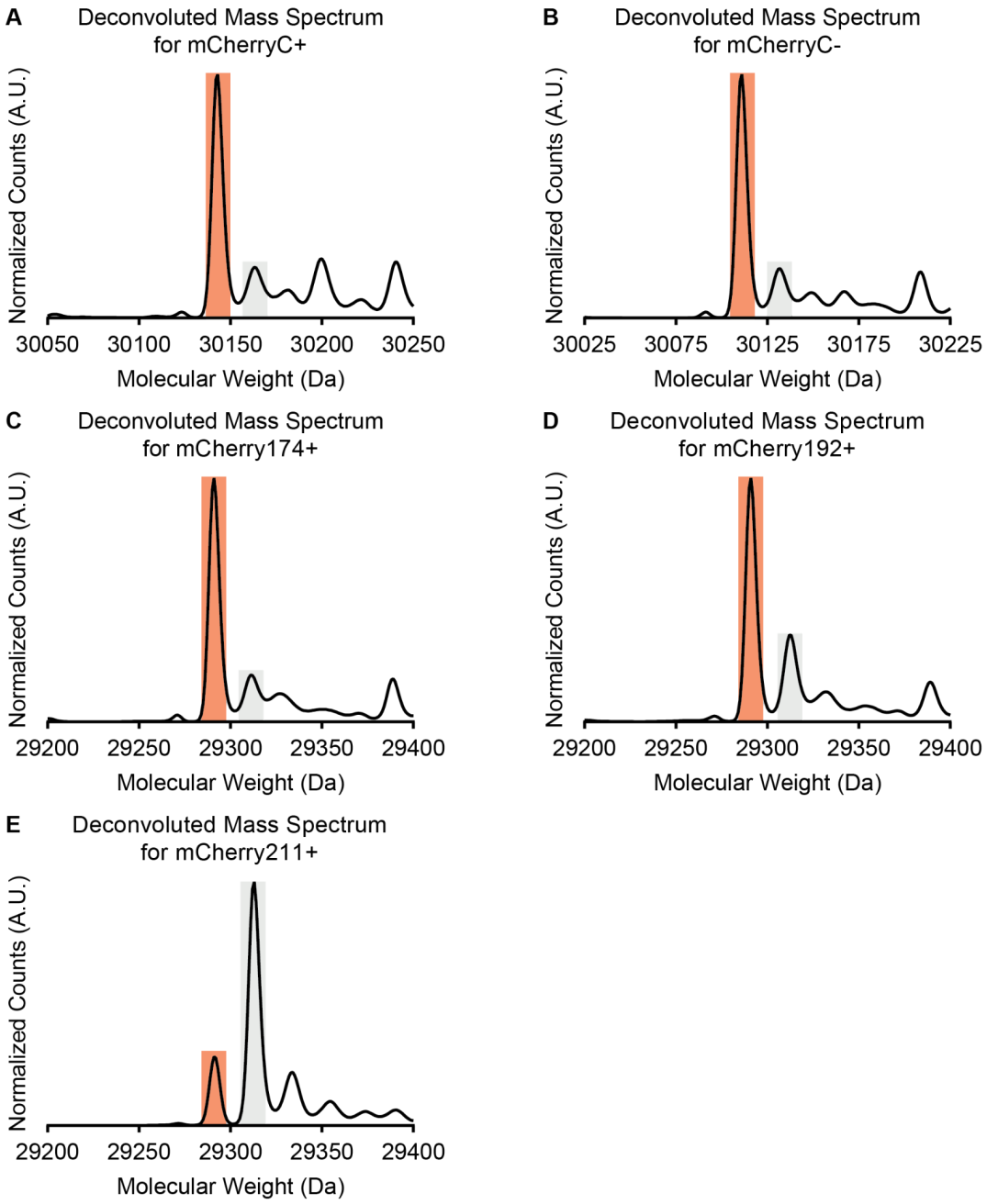
Supplementary Figure 8. Effect of pH on one-pot cyclodehydration and dehydrogenation of peptides containing non-α-amino acid monomers at the -1 site. (A) MicD-F and ArtGox reactions of substrates with non-α-amino acid monomers immediately C-terminal to the site of cyclization. (B) Yields of thiazoline 5(a-i) and thiazole (6(a-i)) products obtained for polypeptide substrates with non-α-amino acid monomers at position -1, immediately C-terminal to the reaction site. (C) Extracted ion chromatograms illustrating the products of MicD-F and ArtGox-catalyzed reactions.



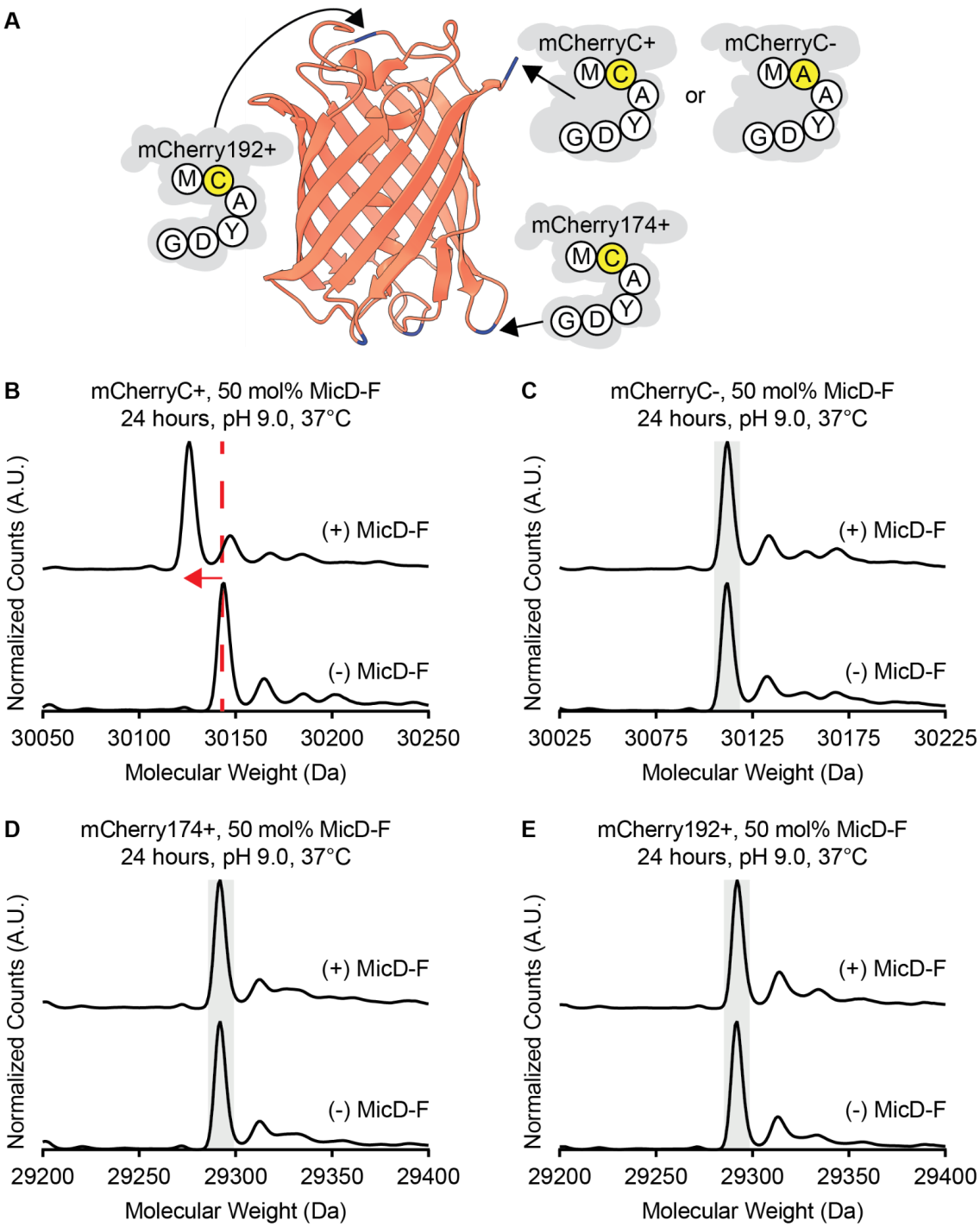
Supplementary Figure 9. Tolerance of non-α-amino acid backbones at the site of cyclization. (A) Cyclodehydration reaction of substrates ITAYDG and IXAYDG (where X = L- or D- ^{3}-threonine) with 50 mol% MicD-F at pH 8.0 or pH 9.0 and 37°C for 24 hours. These conditions led to significant cyclodehydration of the model substrate ITAYDG. However, no cyclodehydration of substrates containing a ^{3}-amino acid was observed at either (B) pH 8.0 or (C) pH 9.0.



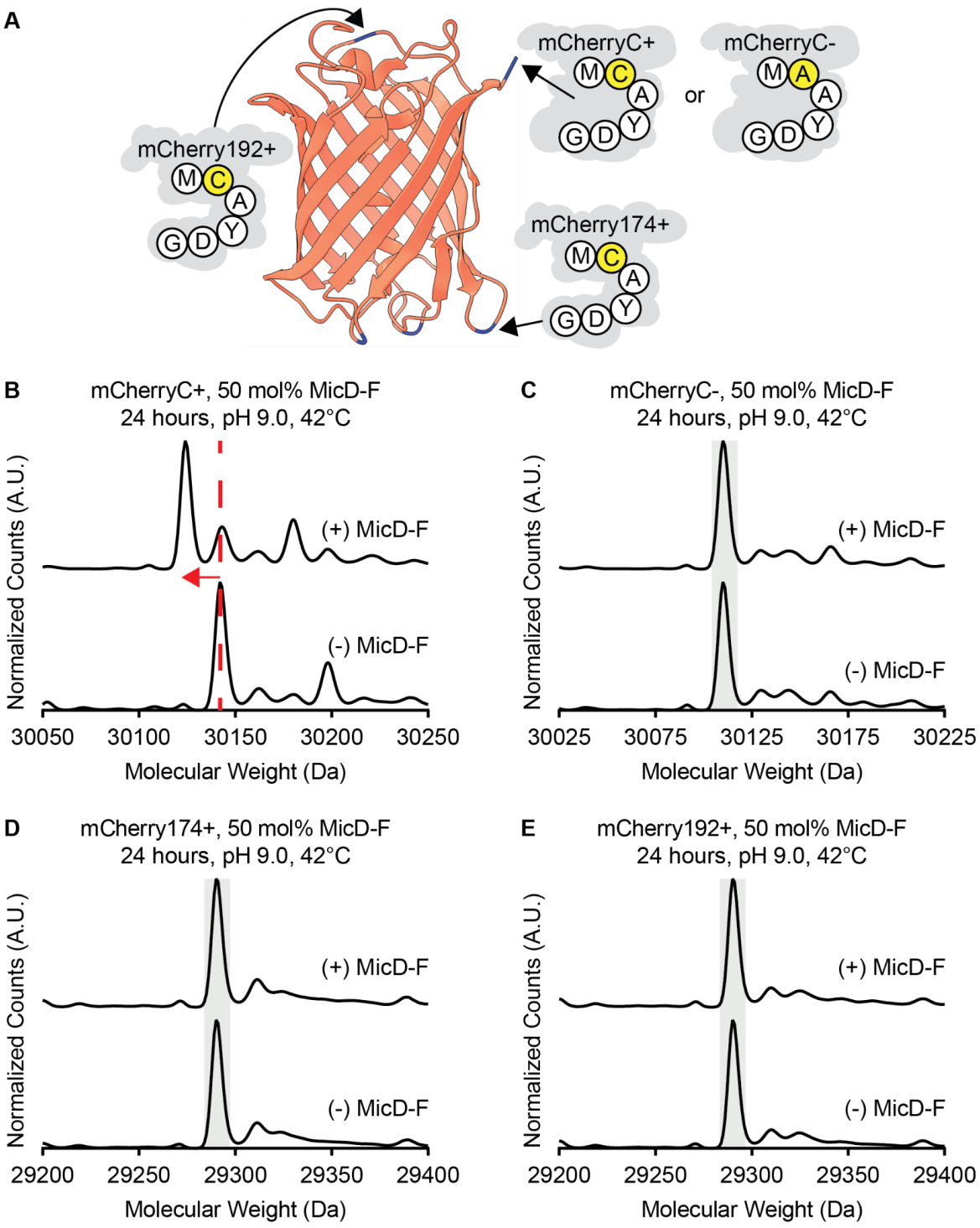
Supplementary Figure 10 Characterization of mCherry variants purified via Talon affinity chromatography. SDS-PAGE analysis of 4 µg of protein separated using a 4-15% mini-PROTEAN TGX gel run at 200 V for 30 minutes. Total protein content was visualized via Coomassie Blue staining. As previously reported,[10,11] boiling of the mCherry constructs for SDS-PAGE analysis yielded two lower molecular weight fragment bands due to backbone hydrolysis. Band intensities were quantified using Bio-Rad Image Lab Software (Hercules, CA).



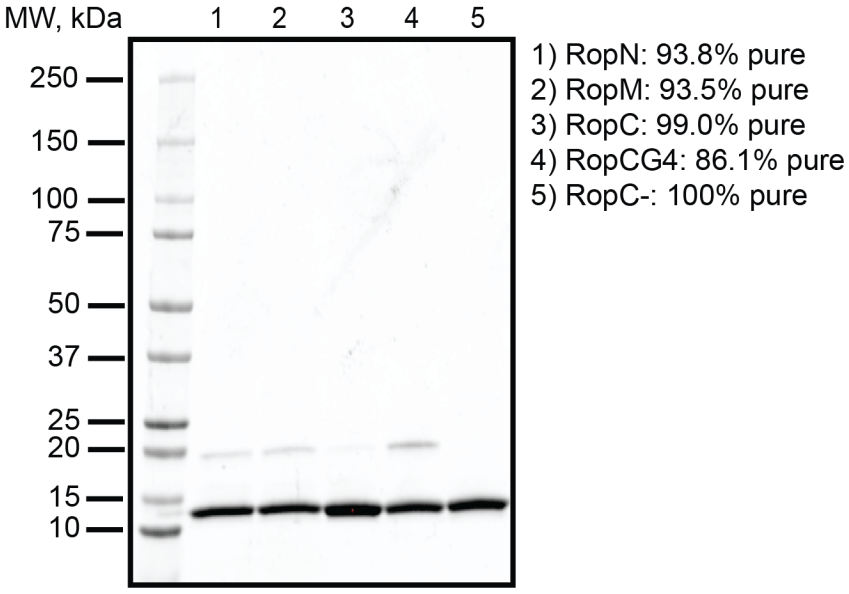
Supplementary Figure 11 Characterization of mCherry variants purified via Talon affinity chromatography. Deconvoluted mass spectrum of (A) mCherryC+, (B) mCherryC-, (C) mCherry174+, (D) mCherry192+, and (E) mCherry211+. Parent protein is shaded in grey and mature protein containing the mCherry chromophore (-22 Da) is shaded in red. The mCherry211+ variant, which displayed poor chromophore maturation, was excluded from further analysis due to presumed folding defects.



Supplementary Figure 12 Cyclodehydration of (A) mCherry variants upon treatment with 50 mol% MicD-F (pH 9.0, 37°C) for 24 hours. Under these conditions (B) mCherryC+ was nearly completely converted to the cyclodehydration product while (C) mCherryC-, (D) mCherry174+, and (E) mCherry192+ gave no evidence of cyclodehydration.



Supplementary Figure 13 Cyclodehydration of (A) mCherry variants upon treatment with 50 mol% MicD-F (pH 9.0, 42°C) for 24 hours. Under these conditions (B) mCherryC+ was nearly completely converted to the cyclodehydration product while (C) mCherryC-, (D) mCherry174+, and (E) mCherry192+ gave no evidence of cyclodehydration.



RopN Sequence
MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELMCAYDGGGGGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

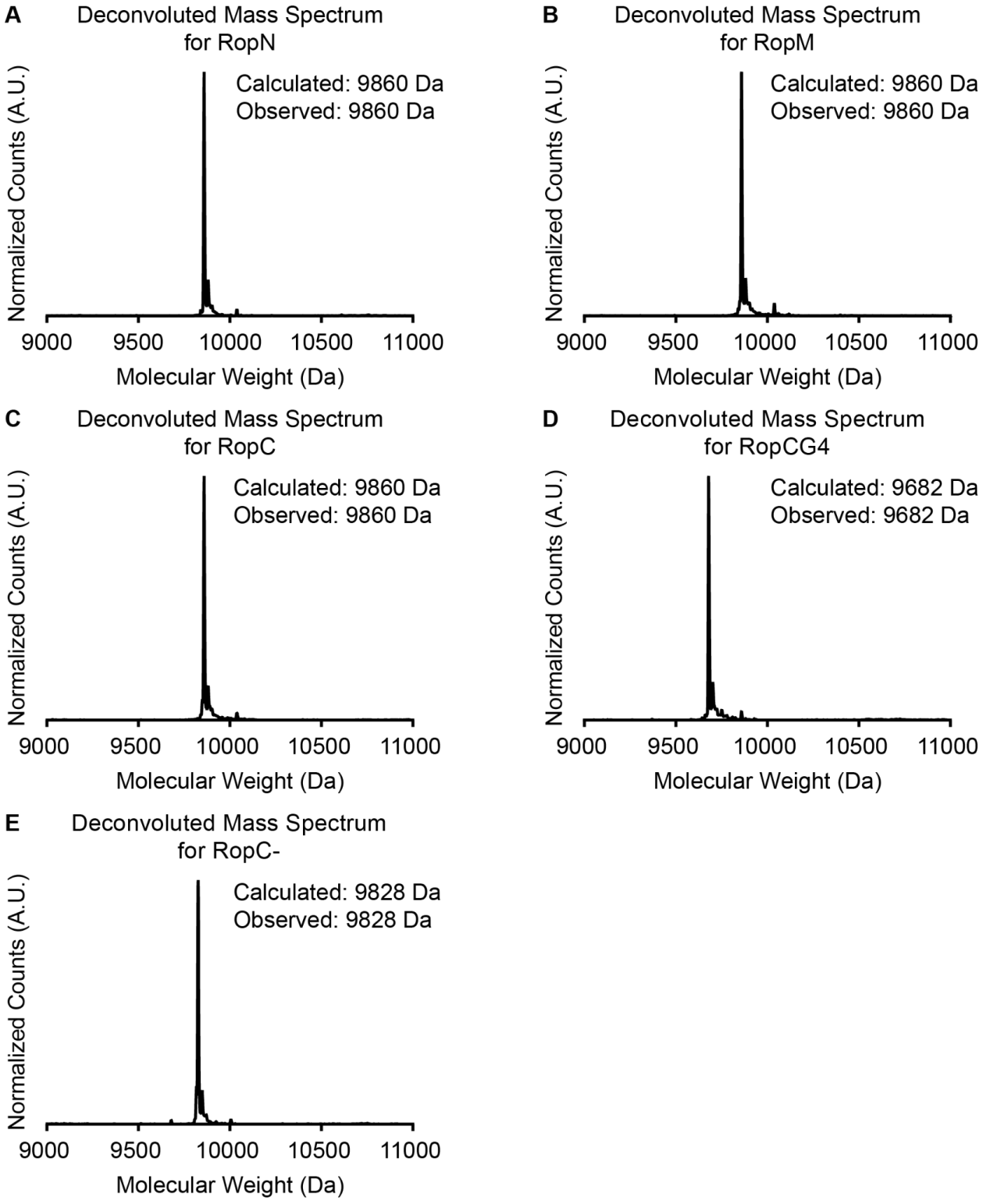
RopM Sequence
MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGMCAYDGGGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

RopC Sequence
MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGGGMCAYDGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

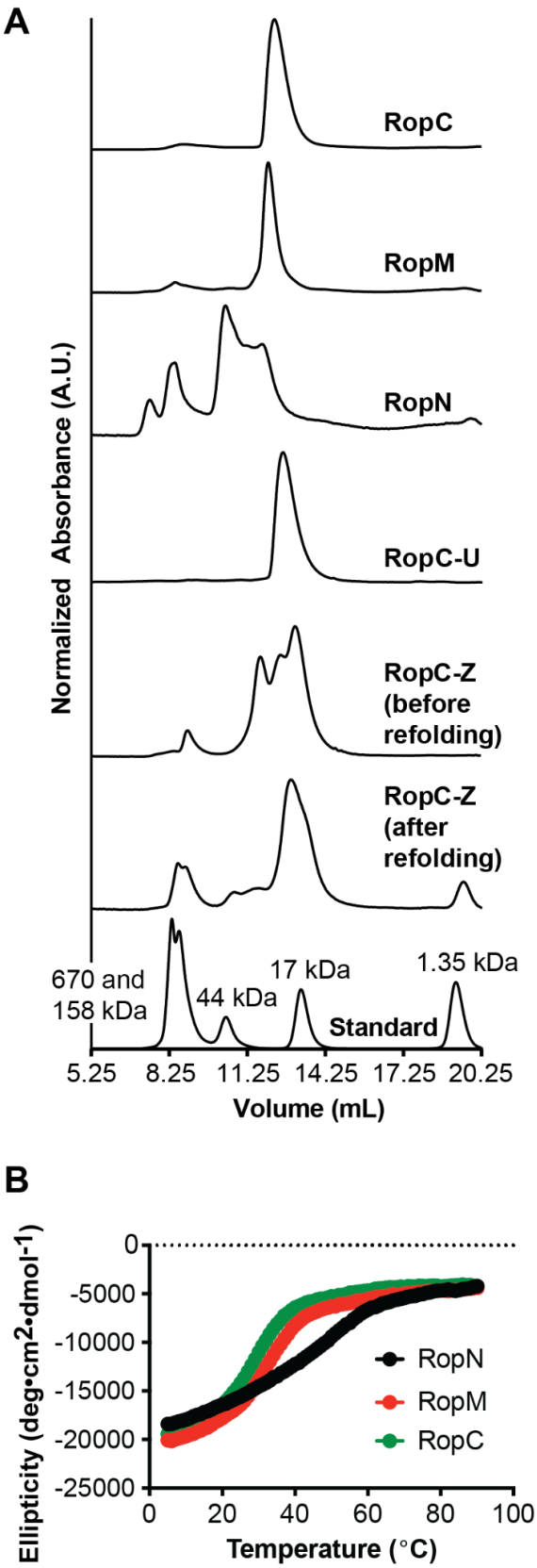
RopCG4 Sequence
MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGGGMCGGGGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

RopC- Sequence
MDYKDDDDKMTKQEKTALNMARFIRSQTLTLLEKLNELGGGGMAAYDGDEQADICESLHDHA
DELYRSCLARFGDDGENLHHHHHH

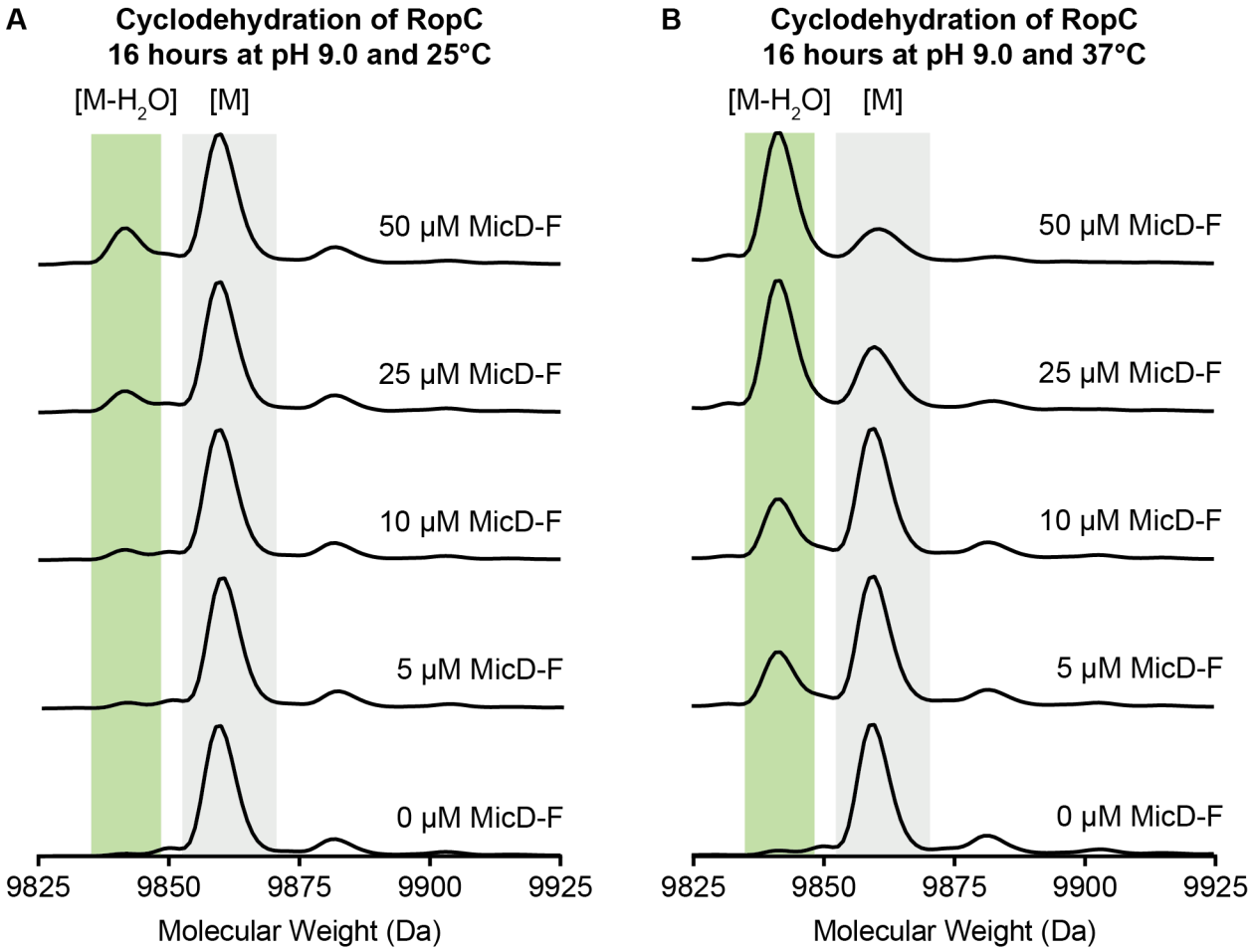
Supplementary Figure 14. Characterization of Rop variants purified via Talon affinity chromatography. All contained an N-terminal **FLAG** purification tag, and one of five **ten-amino acid sequences** in place of the native Asp30-Ala31 sequence of Rop. They also all contained a C-terminal **6xHis** purification tag. SDS-PAGE analysis of 4 µg of protein separated using a 4-15% mini-PROTEAN TGX gel run at 200 V for 30 minutes. Total protein content was visualized via Coomassie Blue staining. Band intensities were quantified using Bio-Rad Image Lab Software (Hercules, CA).



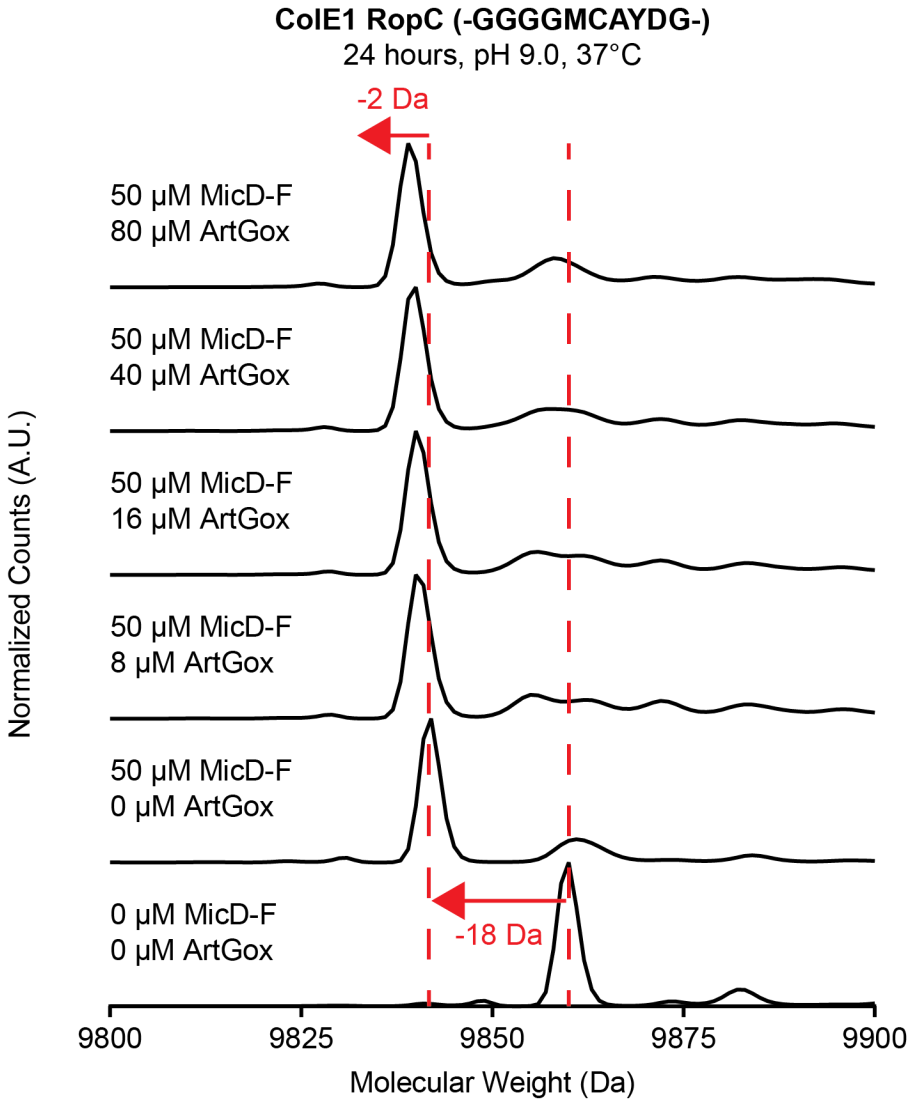
Supplementary Figure 15. Characterization of Rop variants purified via Talon affinity chromatography. Deconvoluted mass spectrum of (A) RopN, (B) RopM, (C) RopC, (D) RopCG4, and (E) RopC-.



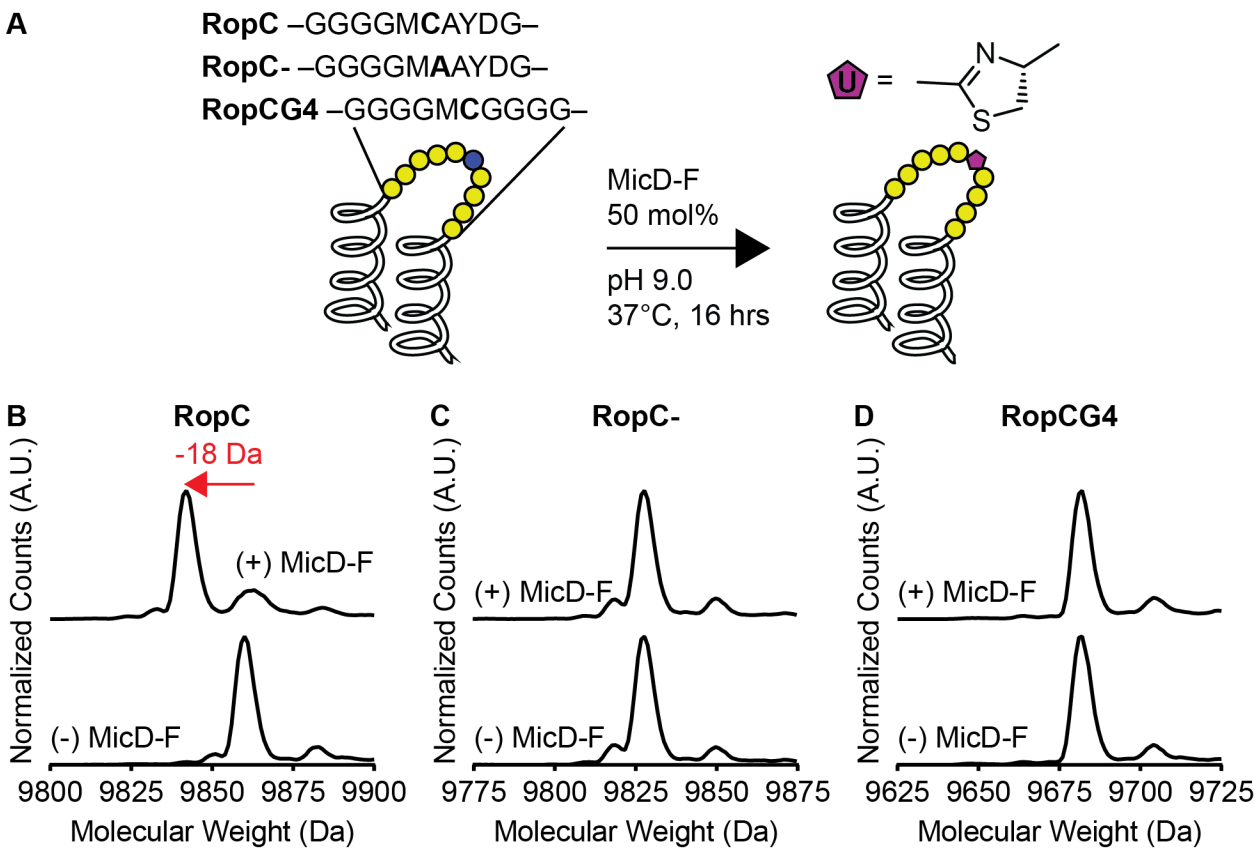
Supplementary Figure 16. (A) Size-exclusion chromatography (SEC) traces for purified samples of RopC, RopM, RopN, RopC-U, and RopC-Z both before and after refolding. Note that RopC-Z remains heterogeneous even after temperature-induced refolding. (B) Temperature dependent CD analysis of RopN, RopM, and RopC.



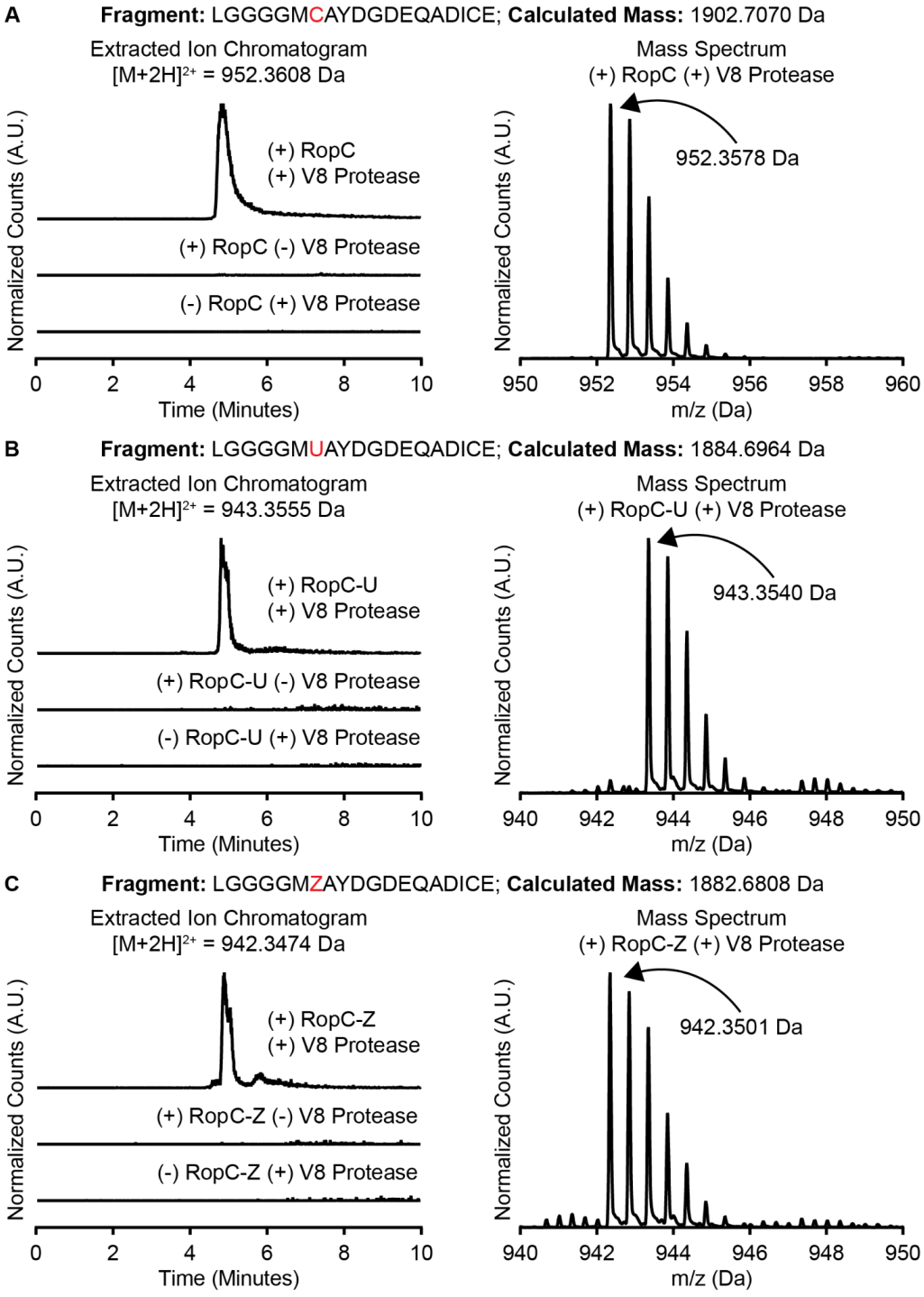
Supplementary Figure 17. Optimizing conditions for the cyclodehydration of RopC by MicD-F. (A) Effect of [MicD-F] on the conversion of RopC (grey bars) into RopC-U (green bars) after 16 h at 25°C. Conversion was minimal even at the highest [MicD-F] used (50 μM). (B) Effect of [MicD-F] on the conversion of RopC (grey bars) into RopC-U (green bars) after 16 h at 37°C. Conversion was highest at the highest [MicD-F] used (50 μM).



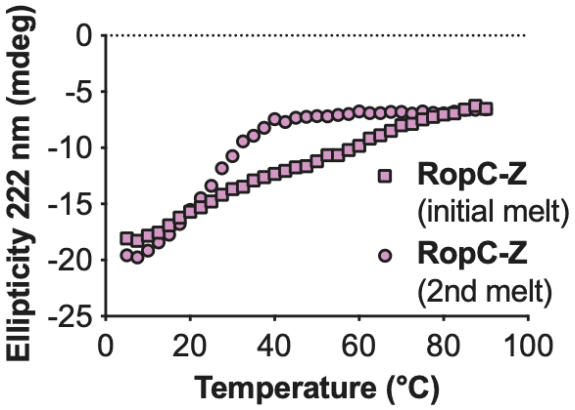
Supplementary Figure 18. Optimization of one-pot cyclodehydration and dehydrogenation of RopC by MicD-F and ArtGox. The deconvoluted mass spectrum indicated that reaction with 50 mol% MicD-F and 80 mol% ArtGox for 24 hours at pH 9.0 and 37°C was sufficient for complete conversion to the thiazole-modified protein.



Supplementary Figure 19. Cyclodehydration of RopC is Cys44-specific and requires the C-terminal AYD recognition sequence. A) Control Rop constructs, RopC- and RopCG4, were designed to test the importance of Cys44 and the C-terminal AYD recognition sequence in cyclodehydration of RopC by MicD-F. B) RopC demonstrated near complete cyclodehydration upon treatment with 50 mol% MicD-F for 16 hours at 37°C and pH 9.0. C) RopC-, which is a cysteine to alanine mutant of RopC, displayed no cyclodehydration under the tested reaction conditions. D) RopCG4, which substitutes the C-terminal AYD recognition sequence with a triglycine sequence, also displayed no cyclodehydration under the tested reaction conditions.



Supplementary Figure 20. Protease digest of RopC, RopC-U, and RopC-Z via V8 protease (endoproteinase Glu-C) from *Staphylococcus aureus* V8 is consistent with enzymatic modification at Cys44. A) LC-MS analysis of RopC digest identifying a fragment containing the MicD-F/ArtGox substrate MCAYDG. B) LC-MS analysis of RopC-U digest showing the expected 18 mass unit decrease relative to the fragment from RopC. C) LC-MS analysis of RopC-Z digest showing the expected 2 mass unit decrease relative to the fragment from RopC-U.



Supplementary Figure 21. RopC-Z requires refolding to display cooperative unfolding behavior. An initial temperature melt monitoring ellipticity at 222 nm yielded a monotonically increasing melt curve (squares). After returning the sample to 25°C a second temperature melt monitoring ellipticity at 222 nm yielded a two-state transition (circles) characteristic of cooperative unfolding. Data reported (n = 1) is representative of behavior observed in three trials, two of which monitored the initial melt at 208 nm instead of 222 nm.

VIII. Supplementary Tables

Supplementary Table 1. Sequences, calculated, and observed masses of peptide substrates described in this work. Notes: All synthetic peptides were prepared as C-terminal carboxylic acids with a free N-terminus. Predicted and observed masses are reported for the $[M + H]^+$ ion of each peptide.

Peptide	Peptide Sequence	Predicted Mass (Da)	Observed Mass (Da)
+1 site model substrate	ICAYDG	641.2599	641.2671
1a	[Bz]-CAYDG	632.2021	632.2060
1b	[TFBz]-CAYDG	704.1644	704.1688
1c	[Coumarin]-CAYDG	700.1919	700.1954
1d	[2-Abz]-CAYDG	647.2130	647.2173
1e	[5-Methoxy-2-Abz]-CAYDG	677.2236	677.2293
1f	[Apy]-CAYDG	648.2082	648.2134
1g	[β^3-HIle]-CAYDG	655.2756	655.2819
1h	[ACHC]-CAYDG	653.2599	653.2667
1i	[ACPC]-CAYDG	639.2443	639.2480
-1 site model substrate	ICIAYDG	754.3440	754.3555
4a	IC-[β^3-HIle]-AYDG	768.3597	768.3724
4b	IC-[β^2-Dimethyl]-AYDG	740.3284	740.3353
4c	IC-[ACHC]-AYDG	766.3440	766.3537
4d	IC-[ACPC]-AYDG	752.3284	752.3361
4e	IC-[2-Abz]-AYDG	760.2971	760.3067
4f	IC-[5-Methoxy-2-Abz]-AYDG	790.3076	790.3158
4g	IC-[Apy]-AYDG	761.2923	761.2977

Supplementary Table 2. mCherry variants used in this work.

ID	Insertion	Insertion Site	Parent MW (Da)	Mature MW (Da)
mCherryC+	-MCAYDG-	C-terminus	30164	30142
mCherryC–	-MAAYDG-	C-terminus	30132	30110
mCherry137+	-MCAYDG-	D137	29312	29290
mCherry192+	-MCAYDG-	V192	29312	29290
mCherry174+	-MCAYDG-	D174	29312	29290
mCherry211+	-MCAYDG-	E211	29312	29290

Supplementary Table 3. Rop variants used in this work.

ID	Loop Sequence	MW (Da)
RopN	MCAYDGGGGG	9860
RopM	GGMCAYDGGG	9860
RopC	GGGGMCAYDG	9860
RopCG4	GGGGMCGGGG	9682
RopC-	GGGGMAAYDG	9828

Supplementary Table 4. Calculated and observed masses of thiazoline (U)- and thiazole (Z)-modified proteins described in this work.

ID	Calculated Mass (Da)	Observed Mass (Da)
mCherryC+	30142	30144
mCherryC+ (U)	30124	30126
mCherryC+ (Z)	30122	30124
RopM	9860	9860
RopM-U	9842	9842
RopC	9860	9860
RopC-U	9842	9842
RopC-Z	9840	9839

IX. Supplementary References

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