Genetically Selected Cyclic Peptide Inhibitors of AICAR Transformylase

homodimerization**

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Materials and Methods

Materials. All reagents were purchased from VWR Scientific or Sigma-Aldrich Fine Chemicals unless specified otherwise. Restriction and DNA-modifying enzymes were purchased from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technologies. Linear peptides were synthesized at the Hershey Macromolecular Core Facility of the Pennsylvania State University. Plasmid, PCR purification and gel extraction kits were purchased from Qiagen.

Recombinant DNA Techniques. Escherichia coli cultures were maintained in LB broth. DNA manipulations were performed with E. coli DH5α-E (Invitrogen) cells. Plasmids were transformed into E. coli by heat shock or electroporation. All DNA sequencing was performed at the Nucleic Acid Facility of the Pennsylvania State University.

Culture Media and Growth Conditions. Antibiotics were provided at the following concentrations: ampicillin 100 µg/ml; chloroamphenicol 50 µg/ml; kanamycin 50 µg/ml; spectinomycin 50 µg/ml. For chromosomal markers, concentrations of antibiotics were reduced 2-fold. Minimal media A supplemented with 0.5% glycerol and 1 mM MgSO_4 was used for all genetic selections.

Genetic Selection. SICLOPPS libraries were transformed into E. coli strains containing integrated reporter and repressor constructs. Transformants were washed with minimal
media A and plated on minimal media A supplemented with 13 μM L-(+)-arabinose, 2.5 mM 3-amino-1,2,4-triazole, 25 μM kanamycin and 50 μM IPTG. After incubation at 37 °C for 3-4 days, Around 200 surviving colonies were picked and re-streaked onto the same media with and without arabinose. Colonies that displayed an arabinose dependent growth advantage were tested for IPTG dependent inhibition of growth (thus filtering out false positives potentially arising through damage to the expression of SICLOPPS or ATIC fusion). Plasmids from strains that passed the above tests were re-transformed into the original ATIC fusion RTHS strain and re-checked for phenotype retention. The 14 remaining peptides were ranked for their activity by spotting serial dilutions of the corresponding cells onto selective media, allowing the conferred growth advantage to be compared at each dilution level. The variable insert regions on the SICLOPPS plasmids were PCR-amplified, and the DNA sequence of the active peptides determined.

To examine the target specificity of the selected cyclic peptides a new RTHS strain containing 434 repressor DNA-binding domain fusion with the *Saccharomyces cerevisiae* GCN4 leucine zipper (LZ) on its chromosome was constructed. The SICLOPPS plasmid of the ATIC selectants was transformed into the LZ RTHS strain and the activity of each cyclic peptide was ranked by drop spotting as before. Cyclic peptides that specifically disrupt the ATIC homodimer were expected to be inactive in the new LZ RTHS strain. However, should the cyclic peptides confer arabinose dependent growth advantage on the RTHS strain by another mechanism, the selectants would also be active in the new LZ RTHS strain (which is identical to the ATIC RTHS strain except for the LZ homodimer). Five of the 14 selectants incurred a growth advantage (arabinose dependent) on the LZ
RTHS strain, showing a lack of specificity toward inhibiting ATIC dimerization and were therefore discarded.

**Cyclic Peptide Synthesis.** Linear peptide 1a (RYFNVC, 10.0 mg, 12.5 µmol) was coupled onto chemically modified PEGA resin and cyclized as described in ref. [7] (6.3 mg, 8.0 µmol, 64%); m/z (MALDI) found 783.6 [C_{36}H_{50}N_{10}O_8S_1 + H]^+ requires 783.4.

Linear peptide 151 (WMFLNVSG, 10.0 mg, 10.5 µmol) was added to a solution of EDC (6 mg, 3 eq, 31.5 µmol) and HOAt (8.5 mg, 6 eq, 62.7 µmol) in DMF (15 ml). The mixture was agitated at room temperature for 24 hours. The solvent was removed in vacuo, the remaining residue was dissolved in 500 µl of DMF and added drop-wise to 10 ml of diethyl ether. The resulting solid was separated by centrifugation and purified as outlined below (7.2 mg, 7.7 µmol, 73%); m/z (MALDI) found 935.1 [C_{45}H_{62}N_{10}O_{10}S_1 + H]^+ requires 935.4.

Crude cyclic peptides were subjected to reverse-phase chromatography (Partisil C-18 Magnum 9 {length 50 cm; particle size 10µM} ODS-3 columns, Whatman) on a waters HPLC system by using a water/acetonitrile gradient with 0.1% trifluoroacetic acid. Mass Analysis was performed on a Mariner mass spectrometer (PerSeptive Biosystems, Framingham, MA).

**Spectrophotometric Assays.** All assays were performed using a Varian Cary 100 Spectrometer. All reaction mixtures were 500 µl in volume and carried out in 1 cm
pathlength quartz cuvettes at 25 °C. The enzyme used in all of the inhibition studies was the avian ATIC, fused to a N-terminal 6X histidine tag to facilitate purification. The peptides were dissolved in DMSO to a final concentration of 2.5 mM. The concentrations of DMSO used in the assay did not affect the activity of the enzyme.

**AICAR Tfase Assay.** 84 nM of ATIC, 50 µM of 10-f-THF and various quantities of inhibitor were mixed in the assay buffer (32.5 mM Tris-HCl, 25 mM KCl, pH 7.4). The mixture was incubated at 25 °C for 2 min before initiating the reaction by addition of 20 µM AICAR. The reaction was monitored by measuring the increase in absorbance due to formation of tetrahydrofolate at 298 nm.

**IMPCH Assay.** To 100 µM of FAICAR in assay buffer (100 mM Tris-HCl, pH 7.4), 84 nM of ATIC was added. The reaction was monitored by monitoring the increase in absorbance due to the formation of IMP at 248 nm.

**Progress Curve Analysis.** AICAR Tfase assays were conducted as outlined above. The inhibitors were assayed under two conditions, limiting the amount of each substrate. In one case 168 nM of ATIC, 100 µM of 10-f-THF and 20 µM of AICAR was used (limiting AICAR), and in the second case 168 nM of ATIC, 40 µM of 10-f-THF and 100 µM of AICAR was used (limiting 10-f-THF). The reactions were monitored as outlined above, for 50 minutes. Results of progress curve experiments were fit using the program DynaFit,[11] which is based in part upon KINSIM and FITSIM approaches.[14] The data was fitted to the standard inhibition models (non-competitive, uncompetitive, mixed and
competitive) and a model in which the inhibitor binds a monomer of ATIC preventing dimerization. More information on the models used can be found in Figures 10 and 11 (supporting information).

Figure 7: Graphic map of the RTHS plasmids for making repressor fusions; (A) Plasmid pTHCP16, (B) pTHCP16 with ATIC.

Figure 8: Wild type 434 promotor sequence of the RTHS plasmids,
**Figure 9:** The optimal level of IPTG (50 µM) was determined by titration. Reporter gene repression was quantified by β-galactosidase assays.

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_s \left(1 + \frac{[I]}{K_i}\right) + [S]}
\]

**Equation 1:** The competitive inhibition equation

\[
\frac{1}{v} = \left(\frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}}\right) + \frac{[I]}{K_i[S]} \frac{K_m}{V_{\text{max}}}
\]

**Equation 2:** Rearranged competitive inhibitor equation used to determine \(K_i\) of cyclic peptide inhibitors by a linear plot.
Figure 10: Progress rate analysis with respect to f-10-THF; graphic representation and the equation of the best fit by Dynafit, line 1 = no inhibitor, line 2 = 125µM c-1a, line 3 = 250 µM c-1a. M = enzyme monomer, E = enzyme dimer, D = inhibitor, S = substrate, P = product.
Figure 11: Progress rate analysis with respect to AICAR; graphic representation and the equation of the best fit by Dynafit, line 1 = no inhibitor, line 2 = 125 µM c-1a, line 3 = 250 µM c-1a. E = enzyme, D = inhibitor, S = substrate, P = product.