A Chemoenzymatic Approach for Synthesizing Hemoglobin Oligomers

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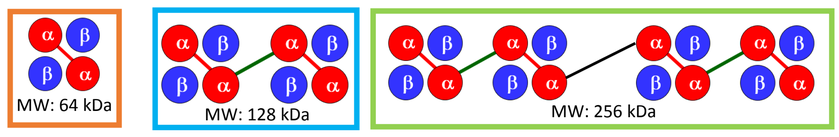
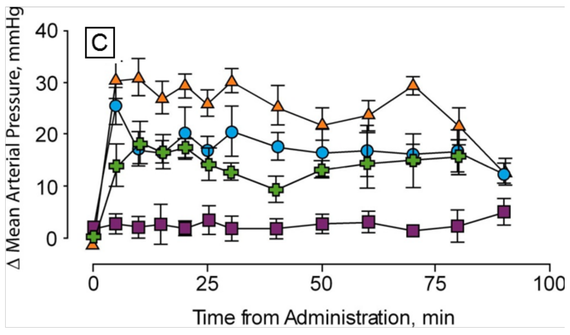
Abstract

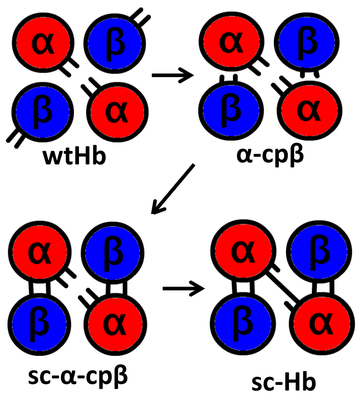
Oligomerized hemoglobin molecules have been shown to decrease previously observed deleterious effects from treatment with cell-free hemoglobin. To create these constructs, a method is developed which employs the site specific ligation reaction of the sortase A enzyme from *S. aureus.* Previously developed Hb mutants (αcpβ) were further modified by adding either the sortase recognition sequence, LPXTG, to the C-terminus of the α-subunit (s-αcpβ), or a tetraglycine motif to the N-terminus (n-αcpβ). Three types of sortase mediated ligation (SML) will be employed in this study. First, we will attempt to ligate Hb subunits directly, using a mixture of s-αcpβ (substrate) and n-αcpβ (nucleophile). The second method will be two-part, first appending either an azide or strained cyclooctyne to the C- and N-termini of the subunits. Using the well established method of Huisgen cycloaddition, the Hb molecules can then be brought together via their chemoenzymatically appended functional groups. To begin with, we will be using doubly modified Hb, and so our products will be a mixture of oligomeric states. In the future, we will use singly modified monomers to begin and cap our oligomers, as well as alternating the addition of azide of cyclooctyne functionalized subunits to better control the oligomeric states of our products.

Background: Hb Mutants



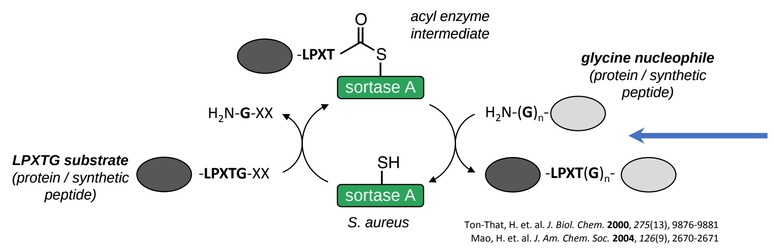


Oligomeric constructs of native Hb have been shown to attenuate the adverse effects of intravenous transfusion of cell free Hb. Here, an octameric Hb construct is shown to attenuate the hypertension response – attributed to its decreased ability to permeate through blood vessels and scavenge nitric oxide.



Rational design scheme of a single-chain Hb polypeptide used by this lab. First, circularly permuting the beta globin, such that the termini are at the interface with alpha. Then, linking alpha and beta through their respective GH helix loops (sc-α-cpβ). Finally, linking native alpha termini together generates sc-Hb.

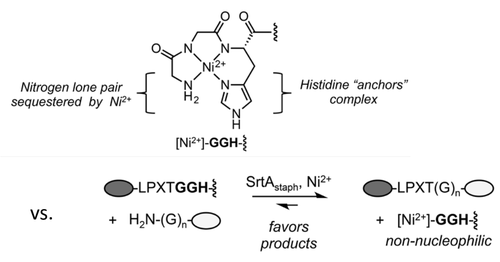
Background: MASML



Regenerates LPXTG motif, susceptible to attack by excised fragment, making the process inherently reversible.

Ton-That, H. et. al. *J. Biol. Chem.* **2000**, *275*(13), 9876-9881
Mao, H. et. al. *J. Am. Chem. Soc.* **2004**, *126*(9), 2670-2671

Including Ni^{2+} in the reaction sequesters excised fragments containing "GGH", reducing reversibility and increasing yield.



Metal assisted sortase mediated ligation has been shown to double yields when ligating synthetic peptides to full sized proteins. The figures to the right depict reactions in which GFP acted as either substrate or nucleophile, and the reconstructed MS spectra showing the increase in product yield when treated with 200 μM Ni^{2+}.

Reaction Conditions:

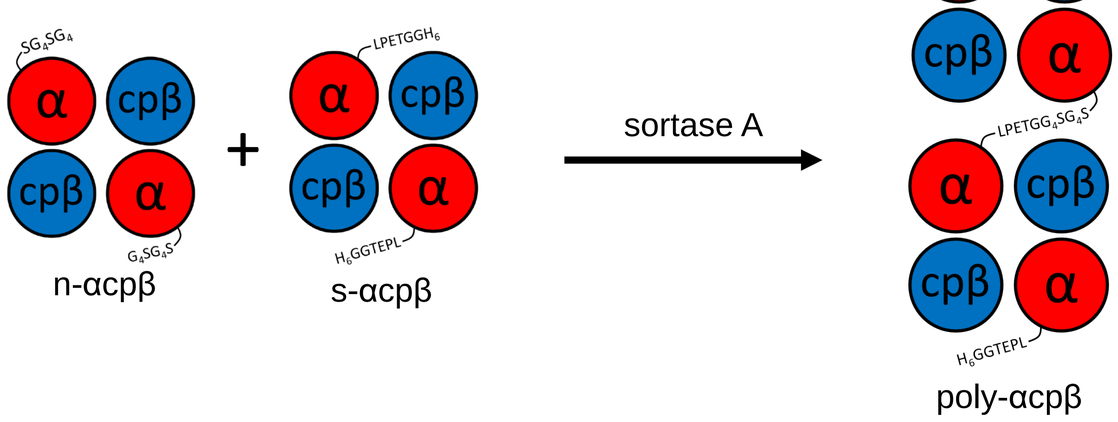
- N-terminal modification of GFP
  - 50 μM nGFP,
  - 50 μM (FITC)Ahx-LPATGGH
  - 20 μM SrtA
  - 200 μM Ni^{2+}
  - Sortase Rxn Buffer
    - 50 mM Tris (pH 7.5)
    - 150 mM NaCl
    - 10 mM $CaCl_2$
- C-terminal modification of GFP
  - 50 μM cGFP,
  - 50 μM GGK(FITC)
  - 20 μM SrtA
  - 200 μM Ni^{2+}
  - Sortase Rxn Buffer
    - 50 mM Tris (pH 7.5)
    - 150 mM NaCl
    - 10 mM $CaCl_2$

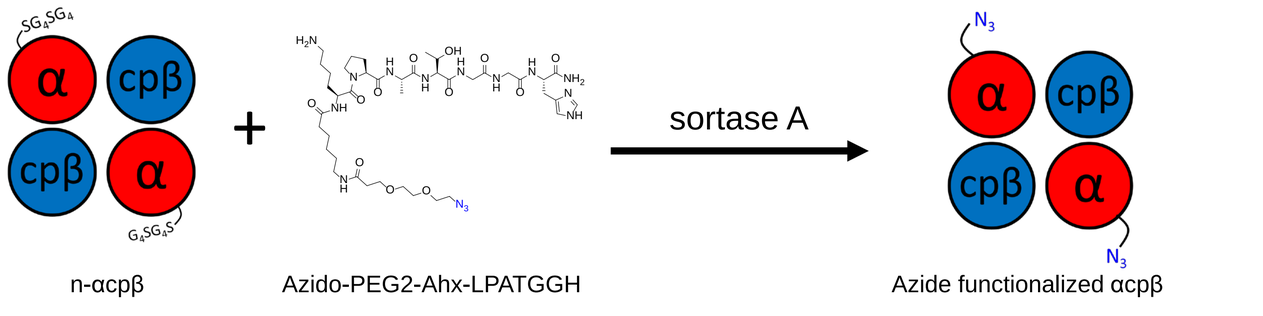
Methods: Hb Modification and Click Handle Synthesis

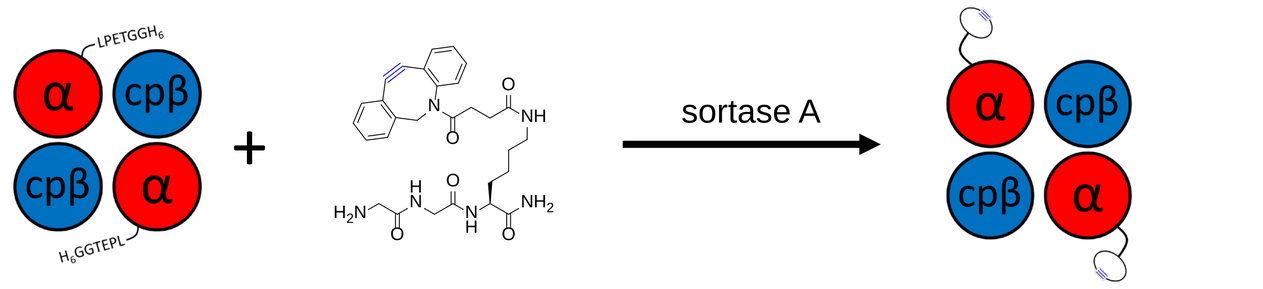
	AcpB with Sortase recognition	Nucleophiles	Substrates
	s-αcpβ	GGK-PEG2-Azido	DBCO-PEG2-Ahx-LPATGGH
	n-αcpβ	GGK-DBCO	Azido-PEG2-Ahx-LPATGGH

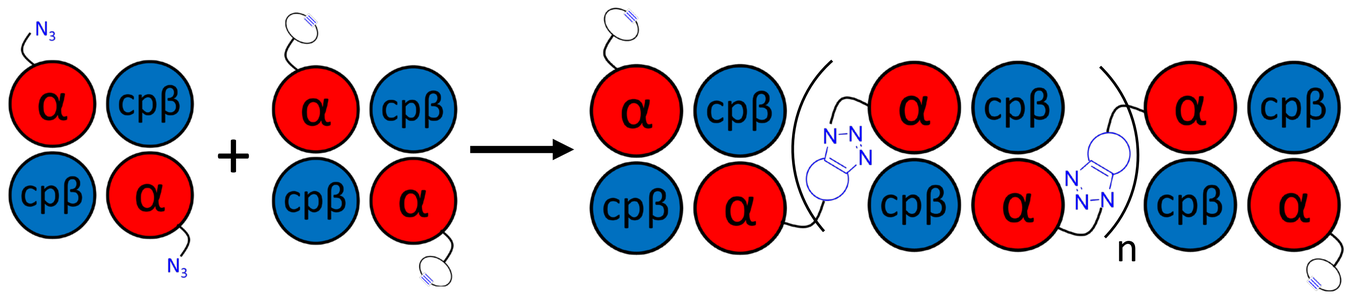
All small peptide substrates and nucleophiles were synthesized using solid phase peptide synthesis. Sortase-functionalized Hbs are recombinantly expressed and purified from *E. coli* BL21(DE3). Gene constructs were manufactured by GenScript.

Methods: Functionalizing Hb Monomers

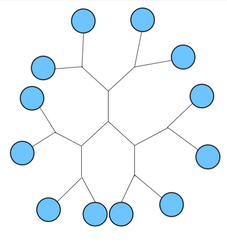








Future Work: Dendrimeric Hb



= azide or alkyne

Acknowledgments

This research was supported by Western Washington University and by WWU Research and Sponsored Programs. The Antos lab acknowledges NIH grant 1RI5GMII9048-01. We also thank Charles Wandler and Dan Carnevale at WWU Scientific Technical Services for assistance with mass spectrometry. As well as, past members of the Antos and Anthony-Cahill Labs.