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14. ABSTRACT

This project concerns the transformation of a de novo designed oxygen transport protein into an effective blood substitute. Second year accomplishments include: (A) The creation and verification of a theoretical model for oxyferrous state lifetimes in oxygen transport proteins. (B) We have modified global protein dynamics to double of oxyferrous state lifetime. (C) We have used surface side chain charges to increase the reduction potential of the bound cofactor and triple the oxyferrous state lifetime. (D) We have incorporated an optimized cofactor binding site and shown that the decrease in protein core dynamics increases the lifetime four-fold. (E) We have made significant progress on the determination of the three-dimensional structure of one of the best performing variants. After a limited amount of additional optimization, this protein is ready for incorporation into the crosslinked particles that are the goal of year three.

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INTRODUCTION

This project concerns the transformation of a de novo designed oxygen transport protein into an effective blood substitute. The protein is less than one-third the size of the human hemoglobin monomer, binds two as opposed to one oxygen-carrying heme cofactor per monomer, and is much more temperature stable than human hemoglobin (*1*). Therefore the protein promises to have a much higher oxygen capacity per unit weight and per unit volume than human hemoglobin. The stepwise goals of the project are to (A) extend the oxyferrous state lifetime by raising the cofactor reduction potential, (B) to optimize the molecular oxygen binding constant to that of human hemoglobin and (C) to create a crosslinked, stabilized preparation of the optimized protein. In the first year of the project, Aim B was completed and some progress was made on Aim A, the most difficult Aim. Below we describe year two progress, which includes major advances in Aim A, progress in Aim C, and the additional engineering of a new enzymatic activity which addresses a major problem which has been raised in the literature subsequent to the start of the project.

BODY

1. Progress toward Aim 1, increasing the oxyferrous state lifetime. The principle obstacle which must be overcome in the creation of this blood substitute is the short (6s) lifetime of the oxyferrous state in the starting protein, which has been named HP7, as opposed to the minutes lifetime of human hemoglobin. We have identified two major differences between our design and natural oxygen carriers which can explain the shortened lifetime:

(A) The low reduction potential of the bound heme cofactor in HP7, -290 mV vs NHE, is approximately 300 mV lower than the values observed for hemes in natural oxygen transport proteins. These low potentials will greatly accelerate the rate of oxidation of the bound heme. An increase in the reduction potential will decrease the rate of oxidation of the reduced cofactor. As oxidized, or ferric, heme complexes are not capable of binding molecular oxygen, slowing the oxidation rate will extend the oxyferrous state lifetime.

(B) The smaller size and higher degree of dynamic motion in HP7 will increase the rate of water penetration into the hydrophobic core of the protein. As proton donation by water is a necessary component of the heme oxidation reaction, slowing the rate of water penetration should further slow heme cofactor oxidation, extending the oxyferrous state lifetime.

1.1 Creating and testing a model for the oxyferrous state lifetime. We have developed a new model for oxyferrous state lifetimes, including an equation which predicts an O_2-concentration dependence. Relevant parameters are the dissociation constant of the oxyferrous complex and the outer-sphere oxidation rate of the uncomplexed ferrous protein:

(1) $$Oxidation\ Rate = k_{ox} \cdot K_d \cdot \frac{([P]+[O_2]+K_d)-\sqrt{([P]+[O_2]+K_d)^2-4[P][O_2]}}{2}$$

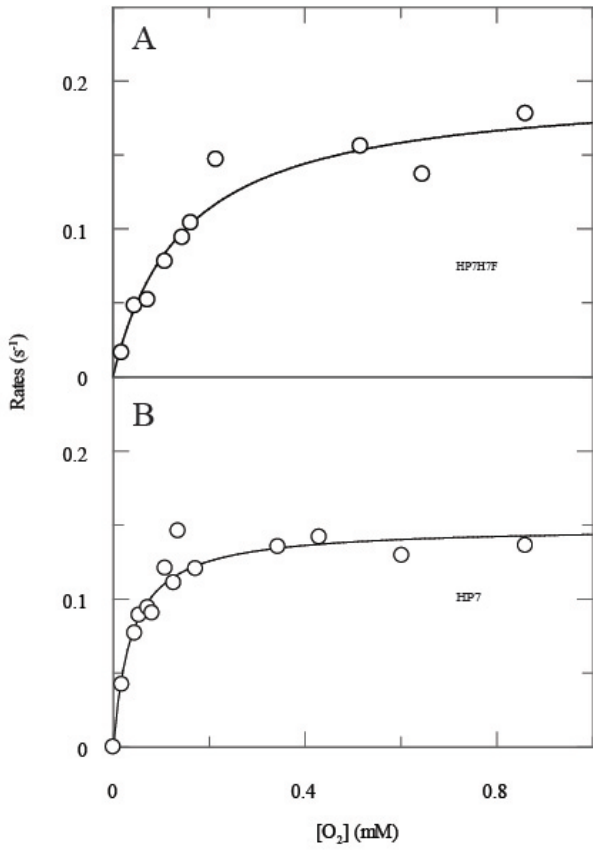

Figure 1. Oxidation of the oxyferrous state of both hemes in HP7 as a function of $[O_2]$. (A) Loop end oxidation. (B) Open end oxidation.

Figure 1 depicts the oxidation rate of the oxyferrous complex of each heme in HP7 measured as a function of the oxygen concentration. The line drawn is a fit with Equation 1 in which the oxygen affinity and the oxidation rate were derived from the best fit to the data. Importantly, the fit oxygen dissociation constant is within error of the value we determined independently using the ratio of the on- and off-rate of oxygen to the ferrous heme-HP7 complex (Figure 2). These results validate our model. A paper has been published in *Biochemistry* reporting these results.

The lifetime is defined as the half-life of the oxyferrous complex at the same oxygen tension as air: 21%. As the oxygen affinity was already optimized in year two, we must slow the rate of k_{ox} under the constraint that we cannot alter this value. As shown below, we have achieved this by raising the reduction potential and by reducing both global and local protein dynamics.

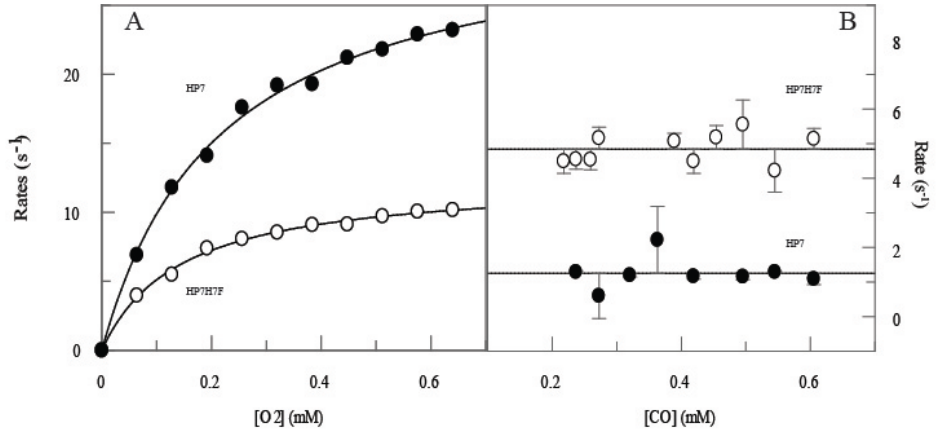

Figure 2. (A) Experimental oxygen on-rates as a function of $[O_2]$. (B) Off-rates determined using double-mixing stopped flow.

1.2 Increasing oxyferrous state lifetime by further limiting water peneration

The oxidation reaction in which a free molecular oxygen collisionally oxidizes a mono- or bis-histidine bound ferrous heme is greatly accelerated by proton donation from water. Water penetration into the hydrophobic core of a protein like HP7 is driven by large-scale or even global protein dynamic motion (*2*). In the first year of this project we demonstrated this by making a series of destabilizing mutations which increased protein disorder and increased water penetration as discerned using NMR-detected hydrogen exchange measurements. Exchange of the backbone amide protons in the hydrophobic core for the unobservable deuterium isotope after dilution of the protein into deuterium oxide is the best method for the quantitation of slow global protein dynamics. Unfortunately, those results were of a binary nature – increased disorder led to such an increase in hydrogen exchange that the rate was too fast to be measured, and at the same time the proteins were unable to detectably bind oxygen. Instead they just rapidly oxidized. We further investigated dynamics in these proteins using Deep-UV raman spectroscopy, and a paper correlating UV-raman signals with dynamics and changes in function in HP7 is in press in the *Journal of Raman Spectroscopy.*

To reduce these dynamics, we took the single heme binding version of HP7, termed HP7-H7F, and made this protein single chain by attaching the two monomers via a glycine-rich loop (helical sequences underlined):

Table 1. Homodimeric and single chain protein sequences

HP7-H7F E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L G G S C S G S G G
E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L

HFHF E I W K Q H E D A L Q K F E E A L N Q F E D L K Q L G G S N S G S G
E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L G G G S T G S G
E I W K Q H E D A L Q K F E E A L N Q F E D L K Q L G G S S G G S G
E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L

This alteration did not alter the reduction potential, heme affinity, pentacoordinate gaseous or ligand binding constant (not shown), proof that the local structure around the heme is unaltered. The two functional features we identified in Q5 as being susceptible to dynamics, however, did change: the distal histidine association constant and the second order oxidation rate constant (see Table 1). The former increases the oxygen dissociation constant even closer to that of human hemoglobin by competing for the ligation site, one of the principle year 2 goals. We thought the latter might be due to a slowing of water penetration due to decreased dynamics.

Table 2 Heme- histidine oxygen binding life time

Protein	ligation	R_{max} (s^{-1})	$K_{d,O2}$ (mM)	k_{ox}($s^{-1}mM^{-2}$)	$t_{1/2}$(s)*
HP7	Open end	0.15±0.01	0.037±0.007	2300±100	7.5±0.1
HP7H7F	Loop end	0.19±0.01	0.15±0.03	2300±50	5.8±0.1

HFHF	Loop added	0.11±0.01	0.09±0.01	1100±100	11.0±0.2

*Oxyferrous state half-life in air (21% O_2).

Hydrogen/deuterium exchange The slower outer-sphere oxidation has been hypothesized to be a result of slower water penetration due to the lower energy for electron transfer when coupled to a proton transfer from water (*3*). Thus we set out to compare the rate of solvent penetration in these two proteins.

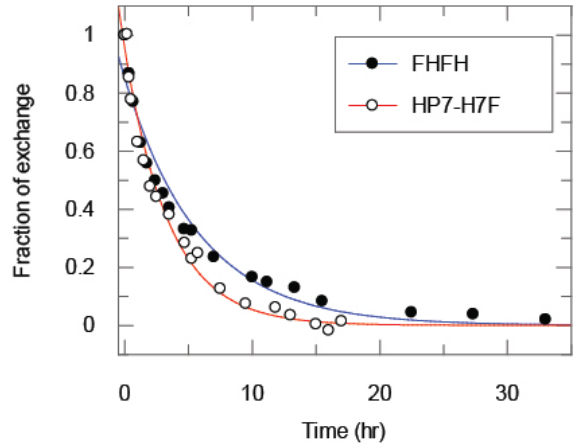

Figure 3. Deuterium exchange data for the two proteins. Hydrogen equilibrated ^{15}N-labelled proteins, each with ferric heme bound, were exchanged into D_2O buffer by passage through a solvent exchange column. 1-Dimensional ^{15}N-HSQC spectra were run at the time points indicated and the total spectral intensity integrated and plotted as a function of time.

Figure 3 depicts the observed rate of solvent exchange in the two proteins. HFHF exchanges 50% slower than HP7-H7F, a rate which agrees, within error, to the increase in oxyferrous state lifetime. This is strong support for the solvent exchange model, which was previously only theoretical. Thus, the transfer to a single chain protein not only increases the oxygen affinity closer to our target value, it further extends the oxyferrous state lifetime almost 50%. A paper describing these results has been submitted.

1.3 Increasing the net surface charge.

We have used surface 'supercharging' to increase the bound heme reduction potential. We created a series of proteins, all of which are based on the protein HFHF, which have surface charge alterations spanning the range from a net charge of -52 to +52. Each protein has the same four helix structure, including loop sequences, as H4. The sequence of each protein had the four repeat alpha helix sequence alterations as follows:

Table 3. Single α Sequences	**Charge**
EIWEQHEEALEEFEEALEQFEELEEL	-52
EIWKQHEDALQKFEEALNQFEDLKQL	-16
EIWKQHEEALKKFEEALKQFEELKKL	0
KIWEQHKKALQEFKKALNQFKKLEQL	+16
KIWKQHQQALKKFQQALKQFQQLKKL	+32
KIWKQHKKALKKFKKALKQFKKLKKL	+52

The +52 variant precipitates randomly, and we have determined that the high lysine and arginine content drives amyloid formation in these proteins by using congo red staining (not shown). The most negatively charged protein folds only in very high salt, and even when folded binds reduced heme too weakly to be an effective blood substitute. The remaining proteins, with charges

ranging from -16 to +32, folded under standard conditions, bound heme tightly in both the oxidized and reduced states, and had bound heme reduction potentials which varied linearly with the net charge (Figure 4).

The heme binding affinities proved to be informative as to natural heme protein function. The bound reduction potential is related to the oxidized and reduced binding affinities and the free heme potential:

(2)
$$E_m^{bound} = E_m^{free} + \frac{RT}{nF} ln \left[\frac{K_d^{Fe(III)}}{K_d^{Fe(II)}} \right]$$

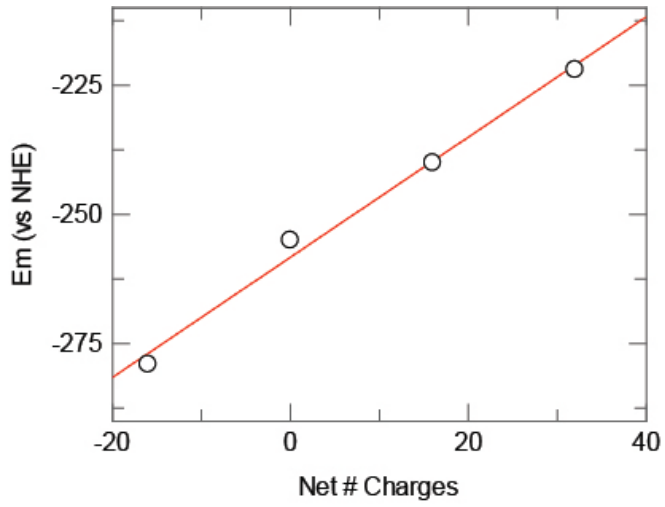

Figure 4. Replot of reduction potentials of the series of supercharged H4 variants.

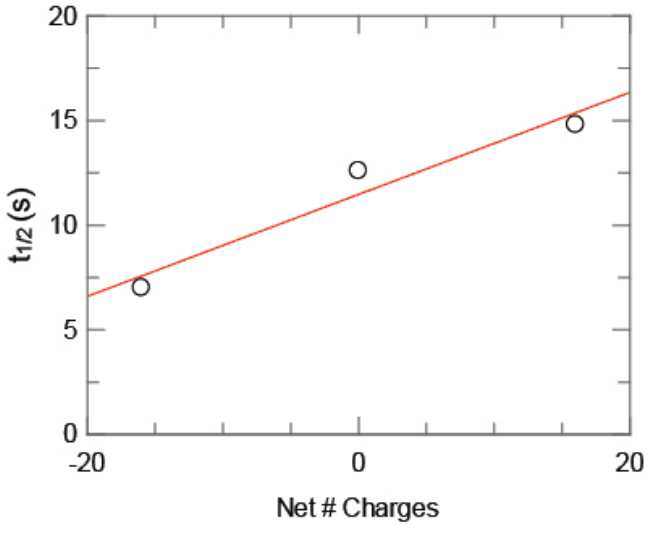

Figure 5. Lifetime of the oxyferrous state increases with the net number of charged residues.

As predicted by this relation, the oxidized binding constant gets weaker as the potential increases, and the reduced constant gets tighter. The fact that the reduced binding constant is too weak for functionality in the case of the -52 variant is explained by this. Because the internal heme binding residues are unchanged in this design series, changes in binding affinities and reduction potentials are the sole result of differences in internal electric fields in these proteins wrought by the surface charges. This result explains the role of the C-type cytochromes in biology – reduction potentials too far away from the free heme potential (-50 mV) in either direction cause binding affinities to be too weak in one of the oxidation states, necessitating covalent attachment. It also sets functional limits on the reduction potentials available in our designed blood substitute. A paper describing this is under preparation.

Oxyferrous state lifetimes in the supercharged variants. As Figure 5 depicts, for the series from -16 to +16 charge, the lifetime almost triples. The +32 protein does not form an oxyferrous

state. The reason has turned out to be relatively obvious – as can be seen in Table 2 the b-position residues, those at functional +32 protein. We have expressed and purified a new +32 protein in which the b-position residues are charged lysine side chains and have shown that this protein does bind oxygen. We are currently determining the kinetic parameters for this variant, and subsequent to that we will submit a manuscript detailing the effects of surface charge alteration on oxyferrous state lifetimes in these proteins.

1.4 The effects of improving packing interactions on protein dynamics and the oxyferrous state lifetime. In the first year of the project we utilized a bioionformatically-derived packing interface, which we published prior to the project start date (*4*), to modify the internal side chains responsible for binding the heme cofactor. These sequence alterations, which we denote as Binding Motif or BM, was placed on both helices which bind the heme cofactor alone or in combination with the mutional removal of the three buried charged residues which drive the hexa- to penta-coordinate ligation equilibrium and thus the opening of a ferrous metal ligation site for oxygen binding. The intent was to further decrease protein dynamics by creating a form-fitting packing interface for the heme cofactor, and therefore further decrease water penetration, extending the oxyferrous state lifetime:

Table 4. BM series protein sequences (loop sequences not depicted, mutations in red)

HFHF 1-3A-	E I L K Q H A D A A I K F A E A L N Q F A D L K Q L
BM-1,3	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L
	E I L K Q H A D A A I K F E E A L N Q F E D L K Q L
	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L
HFHF 3-3A-	E I L K Q H A D A A I K F E E A L N Q F E D L K Q L
BM-1,3	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L
	E I L K Q H A D A A I K F A E A L N Q F A D L K Q L
	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L
HFHF BM-1,3	E I L K Q H A D A A I K F E E A L N Q F E D L K Q L
	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L
	E I L K Q H A D A A I K F E E A L N Q F E D L K Q L
	E I W K Q F E D A L Q K F E E A L N Q F E D L K Q L

As Table 4 demonstrates, this strategy results in a further 4-fold increase in the oxyferrous state lifetime. This is also informational as to the overall topology of the four helix bundle. There are two different ways in which the four linked helices can pack into a bundle with adjacent helices parallel – one results in the b-position glutamates from the first helix buried in the core and serving as the source of potential energy for the hexa- to penta-coordinate conformational change, and one in which the b-position glutamates from the third helix serve this purpose. It is clear from the data that the buried glutamates derive from the first helix.

Table. 5 Heme- histidine oxygen binding life time in the BM variants.

Protein	Sequence modification	$R_{air}(s^{-1})$	$K_{d,O2}$ (mM)	$k_{ox}(s^{-1}mM^{-2})$	$t_{1/2}(s)^*$
HFHF	Original design	0.143±0.005	0.037±0.007	2300±100	11.0±0.2
HFHF_BM1,3	Sequence optimized	0.04±0.01	0.07±0.01	640±90	17±4
HFHF_1_3A_BM1,3	1st helix side chain optimized	0.016±0.003	0.024±0.002	740±70	43±8
HFHF_3_3A_BM1,3	3rd helix side chain optimized	0.04±0.01	0.19±0.04	340±50	18±6

*Oxyferrous state half-life in air (21% O_2) with 1μM protein heme complex.

1.5 Nitric Oxide Dioxygenase activity in H4. This project is modeled after the blood substitute Polyheme – a crosslinked preparation of bovine hemoglobin which was in clinical trials at the time the proposal was written. The trial had an unfavorable outcome because a statistically significant percentage of the study subjects suffered heart attacks or strokes during treatment (*5*). It has since been determined that this is a result of the ability of polyheme to bind the signaling molecule nitric oxide (NO), transport it to other parts of the body, and then release it. NO is a vasodilator, and it is hypothesized that the observed incidents are a result of uncontrolled systemic vasodilation. NO preferentially binds ferric heme, although it has a weak affinity for ferrous heme.

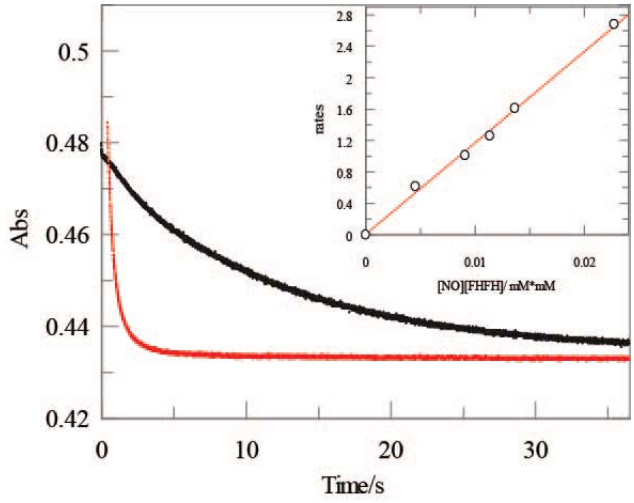

Figure 6. NO dioxygenase activity of H4. Oxidation of ferrous H4 after a double mixing stopped-flow experiment in which the first mix is with O_2-saturated buffer and then the second mix is with degassed buffer (black line) or NO-containing buffer (red line). *Inset:* NO dependence of the oxidation rate.

Mammalian hemoglobins are penta-coordinate: one heme iron ligation site is occupied by a proximal histidine molecule, and one remains open. This site is thus available for nitric oxide binding as well as oxygen binding. However, HP7 was designed to model the hexacoordinate hemoglobins such as human neuroglobin and cytoglobin. These proteins have a destabilized distal histidine which is tightly bound in the oxidized state

and thus unable to bind NO (*6*). Furthermore, they have been shown to be efficient NO detoxification enzymes – the oxyferrous heme can react with NO, resulting in a molecule of nitrate and a ferric heme.

We surmised that our protein, being hexacoordinate, might have a significant advantage over the bovine hemoglobin preparation in that it should not only be incapable of transporting NO, it might serve to eliminate it, therefore greatly reducing the likelihood of cardiovascular problems associated with emergency treatment. As Figure 6 demonstrates, in double-mixing experiments the oxyferrous state of H4 rapidly reacts with NO, with a slope which gives a second order rate constant for NO dioxygenation which is comparable to that found it natural NO detoxification enzymes. There has not yet been a report of a designed enzyme with a catalytic rate which approaches that of natural enzymes. Thus, this activity not only represents a potential considerable improvement over Polyheme, it is a significant advance in enzyme and protein design technology. A paper reporting this has been submitted to a high-profile journal, and we are currently performing additional experiments that the editors have requested.

2. Progress towards a three-dimensional structure of H4-3,3A-BM1,3. The first step in making a cross-linked preparation of the protein is determination of its three-dimensional structure. This enables the identification of surface sites on non-moving parts of the protein which are candidate crosslink sites. Because the protein HFHF_1_3A_BM1,3 is the best performing protein thus far, we have chosen this as the molecule to determine a structure for. We mutated the other site back in, creating H4_1_3A_BM1,3, and then bound two symmetric heme (Fe-protoporphyrin III) cofactors which were synthesized in our lab. The symmetric hemes eliminate problem of insertion isomers while not changing the chemical properties of the cofactor.

We have now assigned the backbone of this protein. 93% of the expected resonances are observable and all of them have been assigned. The majority of the missing resonances are likely lost due to paramagnetic relaxation of nuclei near the ferric heme. Side chain assignments are currently underway.

KEY RESEARCH ACCOMPLISHMENTS

1) We have created and experimentally verified a new physical model for oxyferrous state lifetimes in heme proteins.
2) We have shown that global protein dynamics determine water penetration and oxyferrous state lifetimes, and used this to almost double the lifetime.
3) We have modified surface residues of the protein to raise the reduction potential by almost 60 mV and shown that this modification increases oxyferrous state lifetime threefold.
4) We have modified internal residues to both more tightly bind heme and reduce internal protein dynamics and shown that this increases oxyferrous state lifetimes

fourfold.

5) We have demonstrated that H4 catalyzes NO dioxygenation, a reaction beneficial for its intended purpose as a blood substitute. Catalysis is of the same rate as natural enzymes which catalyze the same reaction – an important first in enzyme design.
6) We have made significant progress in the determination of a three dimensional structure of these molecules. This is the first step in the year three goal – the creation of crosslinked oxygen transport particles.

REPORTABLE OUTCOMES

Manuscripts.

1) Zhang, L., E. M. E. Andersen, Khajo, A, Maggliozzo, R.S., Koder, R.L. (2013). "Dynamic factors affecting gaseous ligand binding in an artificial oxygen transgport protein." Biochemistry **52**: 447-455.
2) Brown, M.C., Mutter, A.C., Koder, R.L., JiJi, R.D., Cooley, J.W. (2013). "Direct quantification of persistent α-helical content and discrete types of backbone disorder during a molten globule to ordered peptide transition." J. Raman Spect. In Press
3) Zhang, L., Koder, R.L. (2013). "Water Penetration Limits the Oxyferrous State Lifetime of an Artificial Oxygen Transport Protein." Submitted
4) Andersen, E.M.E., Zhang, L., Norman, J.A., Brisendine, J.M., Koder, R.L. (2013). "Nitric oxide dioxygenase activity in an artificial hexacoordinate hemoglobin." Submitted
5) Zhang, L., Koder, R.L. (2013). "A bioinformatically optimized heme binding motif increases cofactor affinity and oxyferrous state lifetime in an artificial neuroglobin." In preparation
6) French, C.C., Mutter, A.C., Zhang, L., Everson, B.A., Koder R.L. (2013). "Surface protein supercharging modulates cofactor reduction potentials and oxyferrous state lifetimes in an artificial neuroglobin." In preparation
7) Everson, B.H., French, C.H., Mutter, A.C., Nanda, V., **Koder, R.L.** Hemoprotein Design Using Minimal Sequence Information. Submitted

Presentations.

1) Biophysical Society 57th Annual Meeting, 'Designed Protein Therapuetics', Philadelphia, PA, Feb. 2013
2) Los Alamos National Laboratories New Energy Workshop, 'Designing Artificial Protein Function', Santa Fe, NM, Sept. 2012
3) NYAS 5th Annual Advances in Biomolecular Engineering, 'Minimalist Protein Design and Bioenergy', New York, NY, April 2012

CONCLUSIONS

The creation of an ideal monomeric oxygen transport protein, the goal for years one and two of the project, is nearing completion – the different mechanisms we have determined for extending the oxyferrous state lifetime, when combined, should extend the lifetime well into the minutes time scale. We have demonstrated sufficiently tight cofactor binding in both oxidation states to make a functional biomaterial. We have demonstrated that we can fix the oxygen affinity to values appropriate for a blood substitute.

We have further begun the year three project of creating a cross-linked preparation which is similar to polyheme. The first step in this process is to determine the three dimensional structure of the molecule and use this to identify surface positions on the protein which can be used as anchor points for crosslinks.

Why is this important or "So what": Many different features of the protein have to be near perfect for the blood substitute to work. Tight cofactor binding is critical for a long functional and storage lifetime of a blood substitute. The correct oxygen affinity is necessary for the protein to pick up blood in the lungs and drop it off in the extremities. The bound state must last long enough for circulation to occur. We've engineered all of these features into the designed protein, and along the way have learned some things about natural oxygen transport function. After a limited amount of additional optimization, this protein is ready for incorporation into the crosslinked particles that are the goal of year three.

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