SAND2012-9400C

Enhanced Stabilities and Activities of OPH

Part I: Immobilizations → promote stability and activity

Benefits of:

- **extra active-site metal**
- **crowding reagents**
- **covalent crosslinking**
- **substrate**

Part II: Cell-free production of rational OPH mutants with higher thermal stabilities and activities

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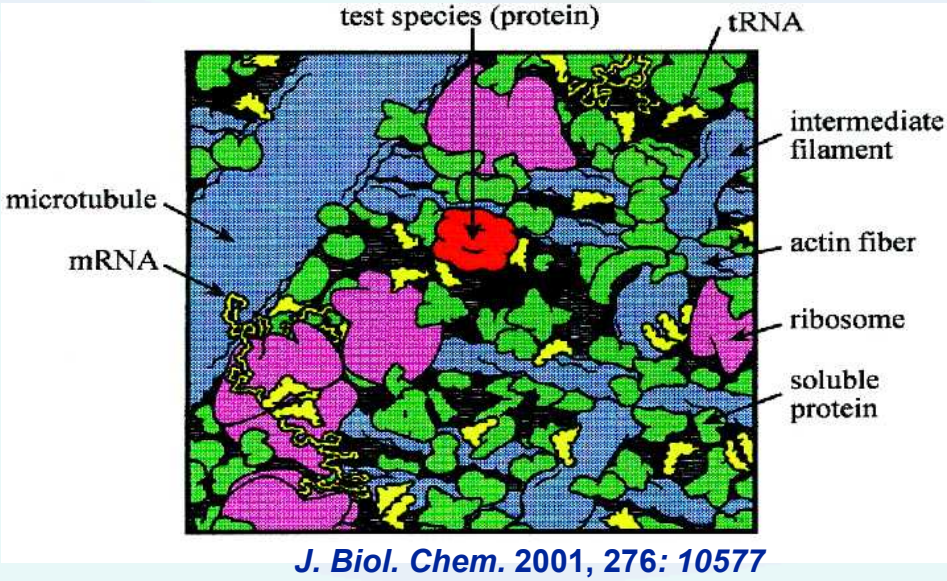
Background: Overcoming enzyme fragility by proper immobilizations

Historical approaches:

- Low levels of immobilized enzyme, and/or
- Immobilization procedure destroys enzymatic activity

Cells are crowded with biomolecules (~400 mg/ml) and structures.



J. Biol. Chem. 2001, 276: *10577*

Inspiration for this work:

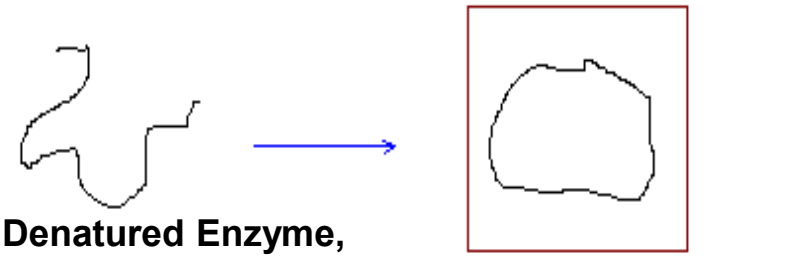
- Cellular architecture
- Molecular crowding
- Rational design of materials
- Rational design of enzymes
- → exceed biological capabilities

Maintaining and Promoting Enzyme Activity

Confinement can eliminate some expanded configurations of the unfolded chain, shifting equilibrium from unfolded (inactive) to native (active).

Cells, molecular crowding, enhancing specific activity

Biochemistry 2001, 40: 11289



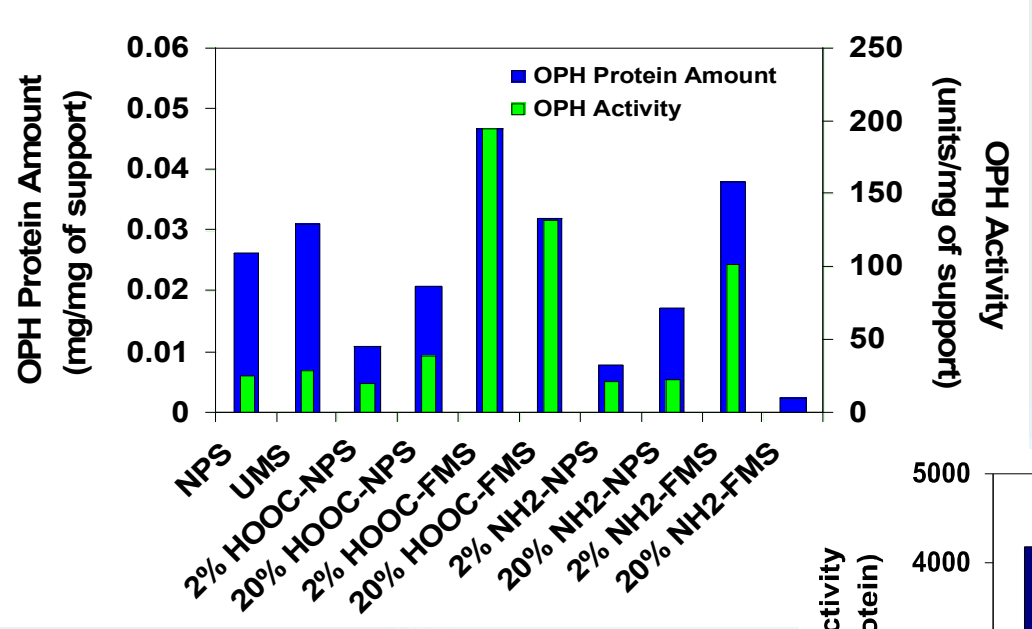
Denatured Enzyme, unfolded state in solution

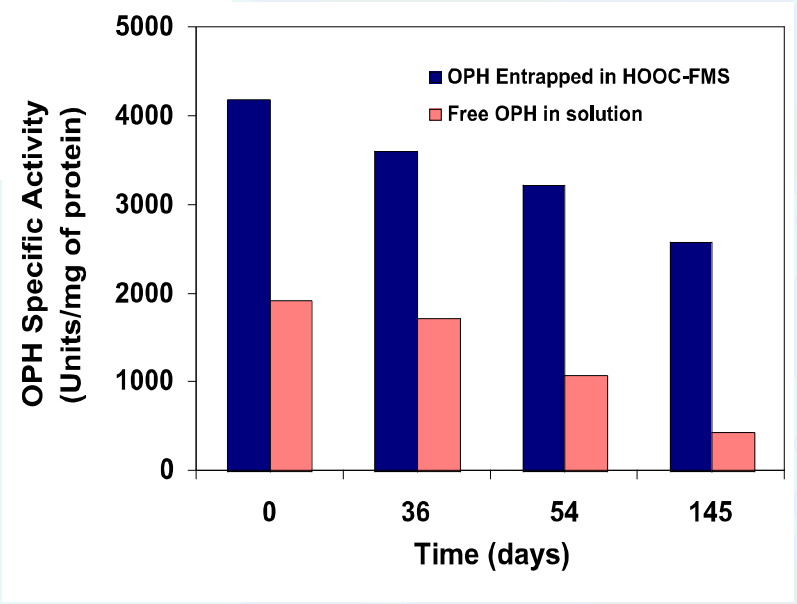
Renatured Enzyme, native state in a confined space

Samples of OPH solution s	Specific activity of OPH solution (units/mg of protein in solution)	Specific activity of OPH after entrapping in FMS (units/mg of protein in FMS)
Sample 1	1629.70	4286. 29
Sample 2	1327.15	2954. 15
Sample 3	2018.03	3375. 89
Sample 4	1494. 77	3789. 28
Sample 5	782. 04	2233. 08
Sample 6	1863. 56	3357. 55

Lei, C. et al. *Nanotech 2006* 17: 5531 and highlighted perspectives by Dunker & Fernandez *Trends Biotech* 25: 189

Comparison of different porous silica support for OPH immobilization

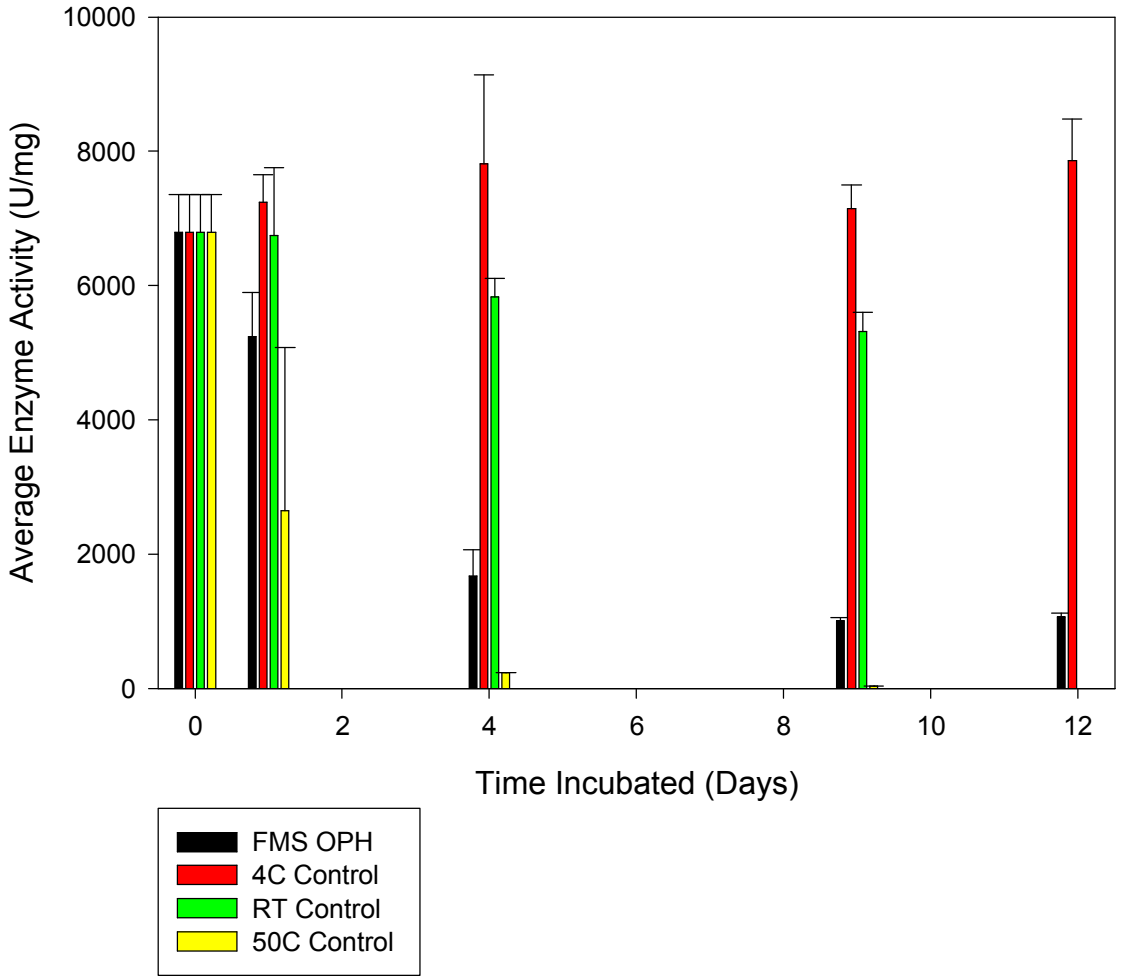


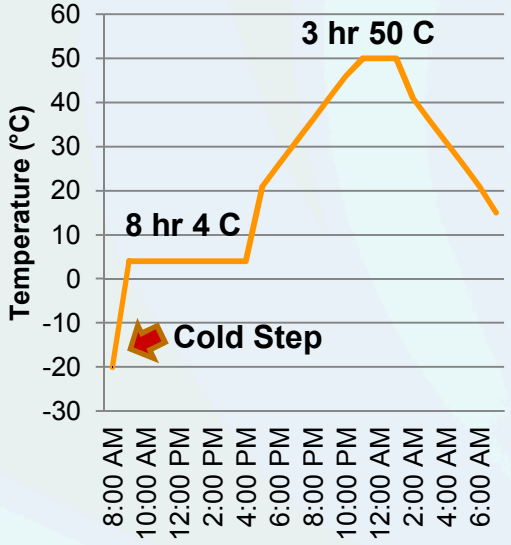


Lei, C., Shin, Y., Liu, J., Ackerman, E. J. 2002. *J. Am. Chem. Soc.* 124 (38): 11242-11243. Highlighted in *Chem. & Eng. News* [80: 35 (2002)] and *Science* [300: 277 & 290-293 (2003)]

Enhanced Specific Activity & Stability of Immobilized OPH

Diurnal Cycling of FMS OPH with -20C Step





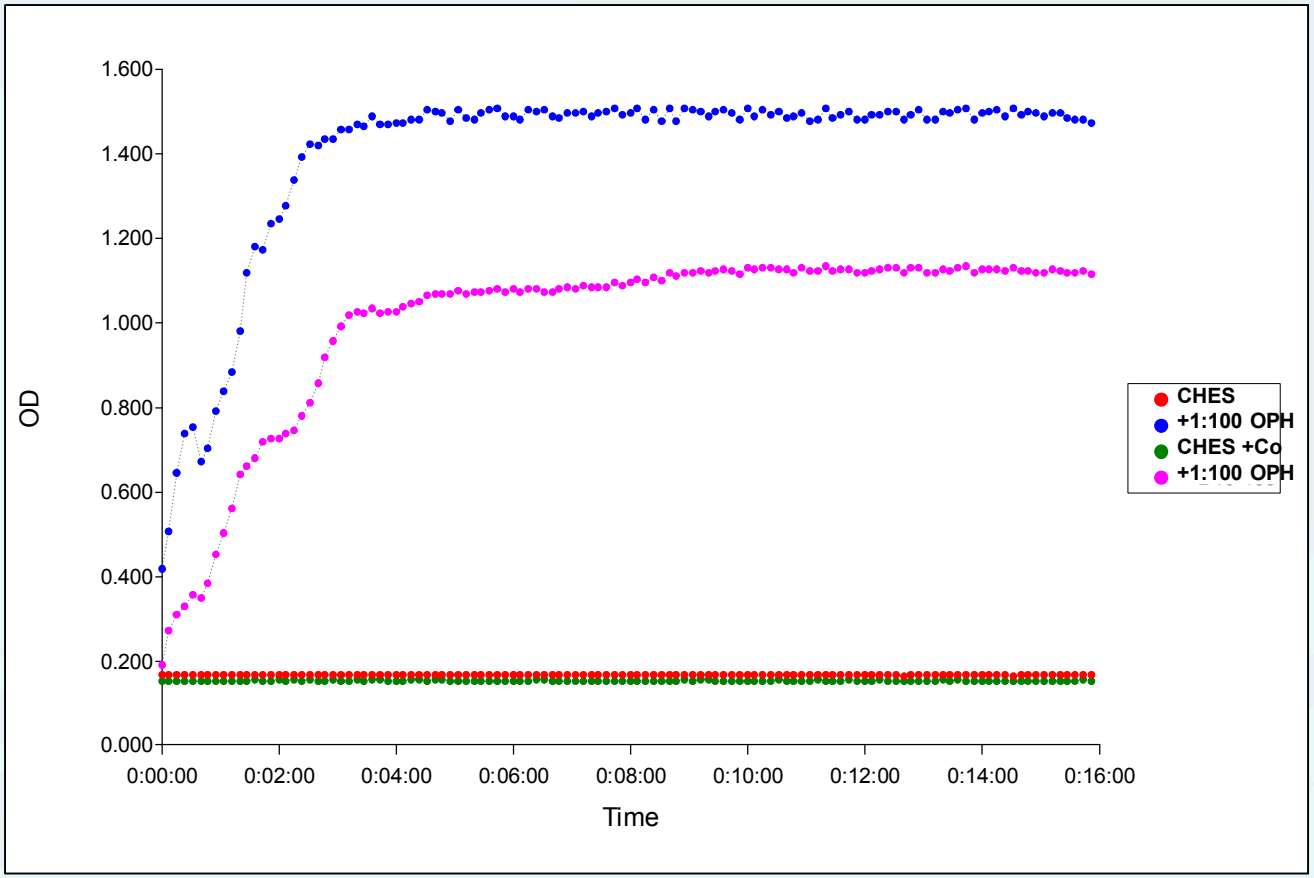
Timeline Diurnal Cycle

Temperature (C)	Hours at Temperature
-20	1
4	8
21	1
26	1
31	1
36	1
41	1
46	1
50	3
41	1
36	1
31	1
26	1
21	1
15	1

Lyophilized OPH stable, but when OPH in solution: FMS OPH > OPH, and

Constant temps > Diurnal cycle. Same with other stressors (pH, peroxides). Detergent inactivates.

OPH activity using plate reader ± 100 uM Co acetate



BioTek Eon Plate Reader
Shaking
Temp up to 60 C
~2 min for 96 samples

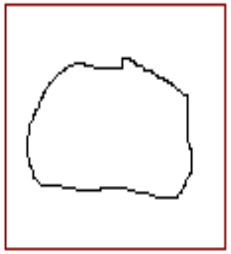
Plate Reader faster, better signal-to-noise than stirred spectrophotometer cuvette.
Extra active-site metal increased activity. (Same for Zn-acetate)

Will it improve stability in FMS by keeping active site in correct conformation?

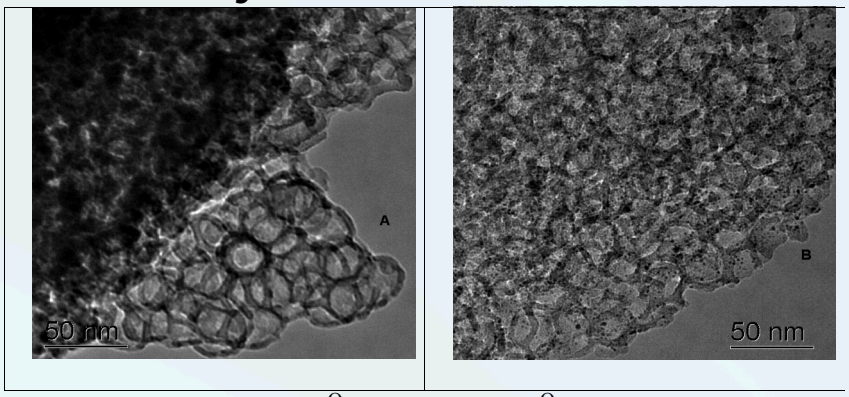
Crosslinking OPH to FMS

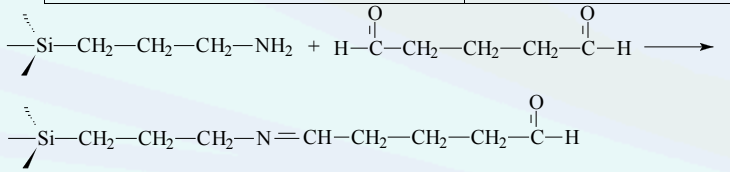
Purpose: Test hypothesis that further immobilization by crosslinking OPH to FMS increases specific activity.

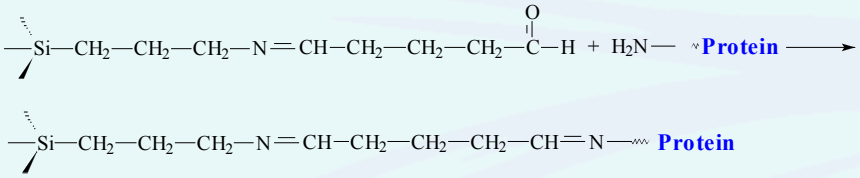
Denatured Enzyme, unfolded state in solution



Renatured Enzyme, native state in a confined space

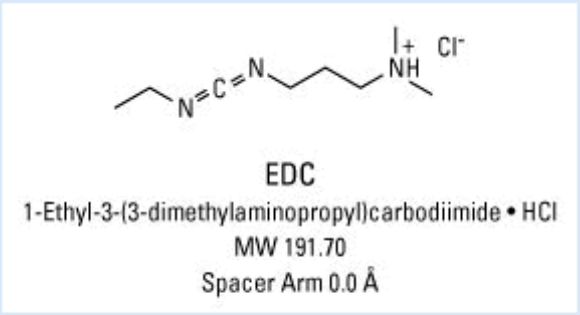


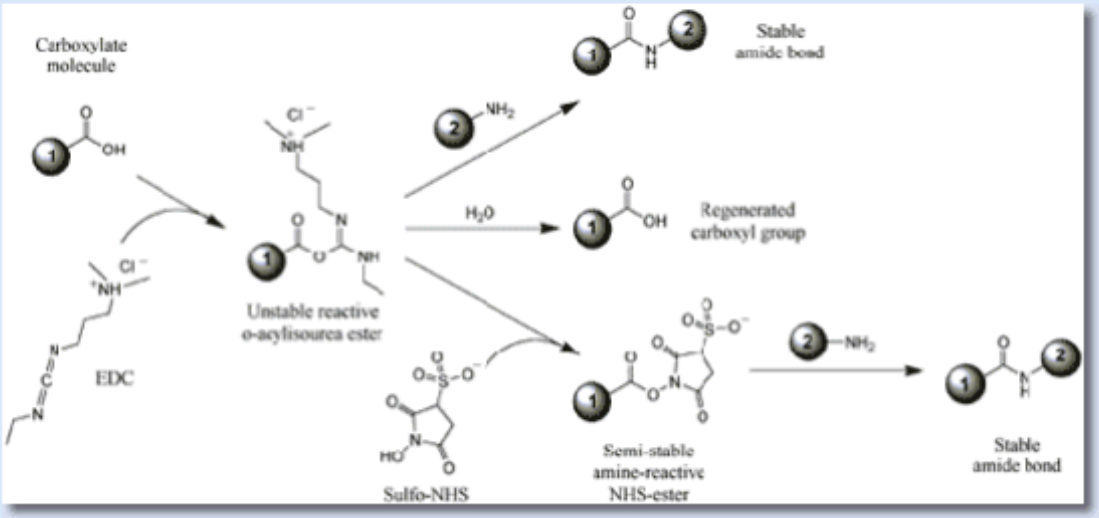




Mesoporous silica can be functionalized with amino, carboxyl, or sulfhydryl groups to provide a favorable environment for proteins.

COOH-NH2 Crosslink via EDC

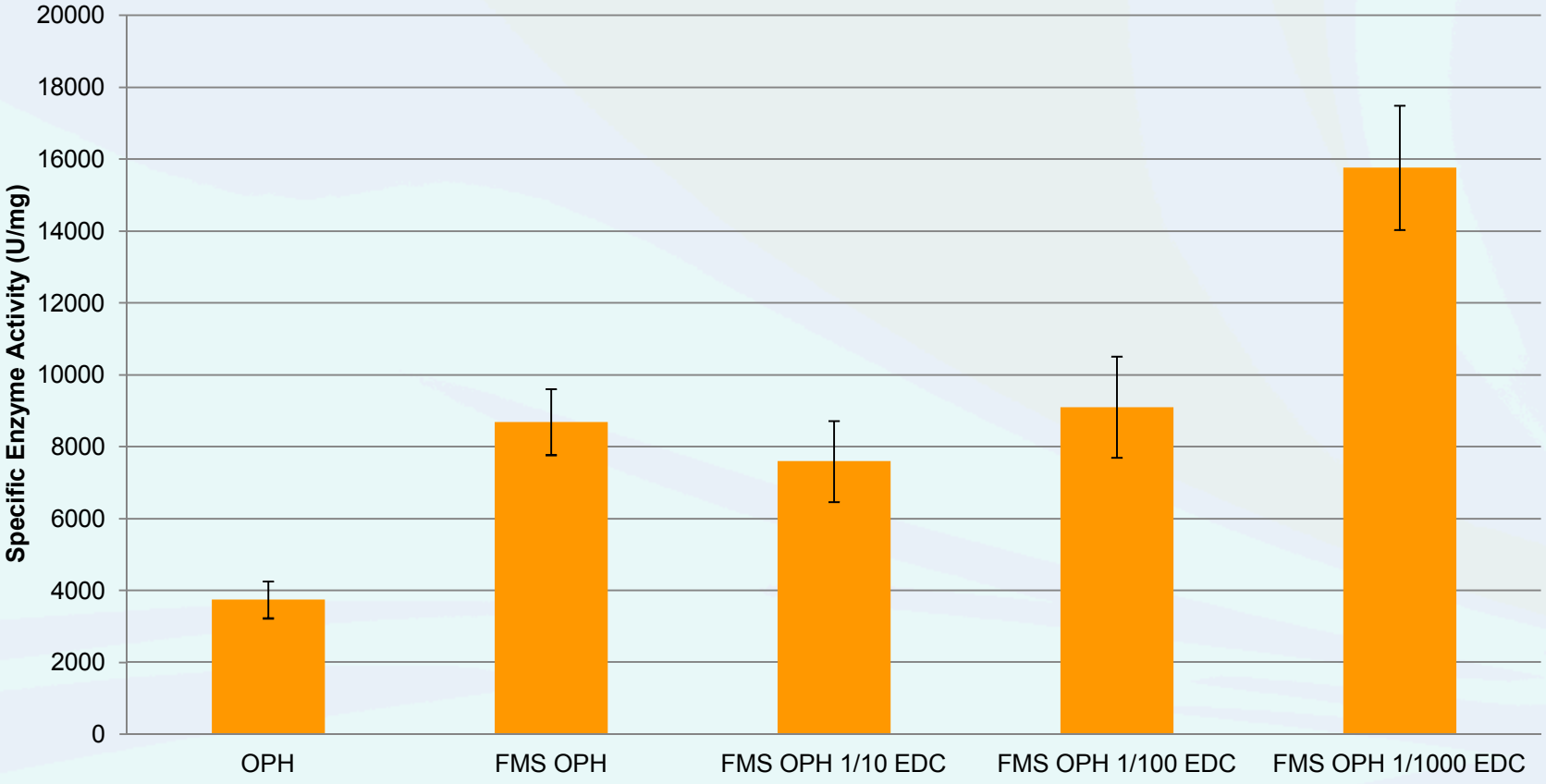




- Zero-length crosslinking agent.
- Couples carboxyl groups to primary amines.
- EDC that does not encounter an amine rehydrolizes to a carboxyl group.
- EDC can convert carboxyl groups to amine-reactive Sulfo-NHS esters in the presence of Sulfo-NHS (N-hydroxysulfosuccinimide)
- Sulfo-NHS improves coupling efficiency.

http://www.piercenet.com/browse.cfm?fldID=02030312

Effect of Crosslinking on OPH Activity

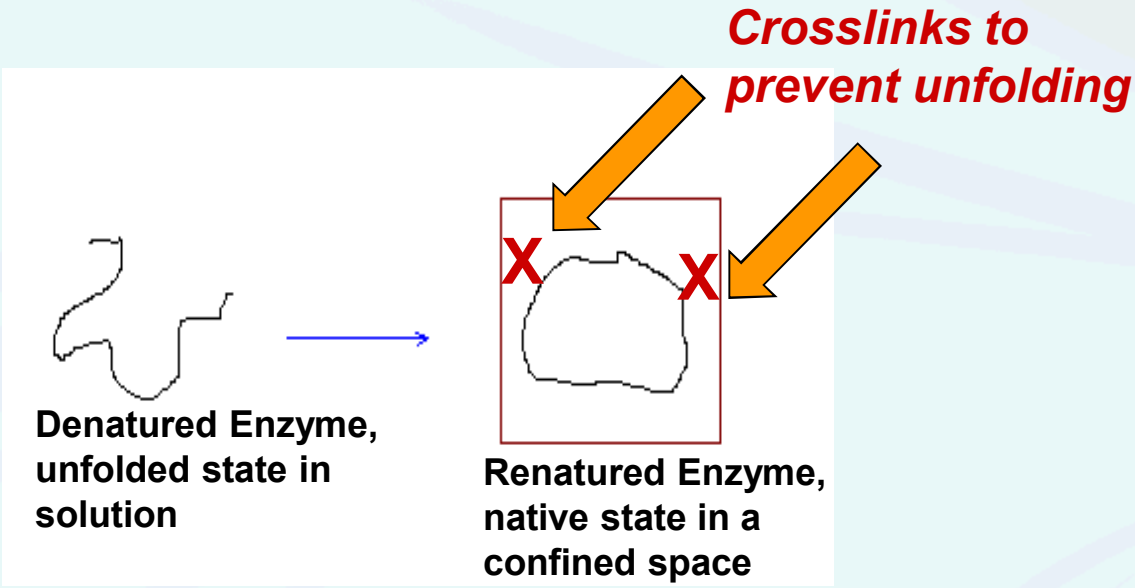


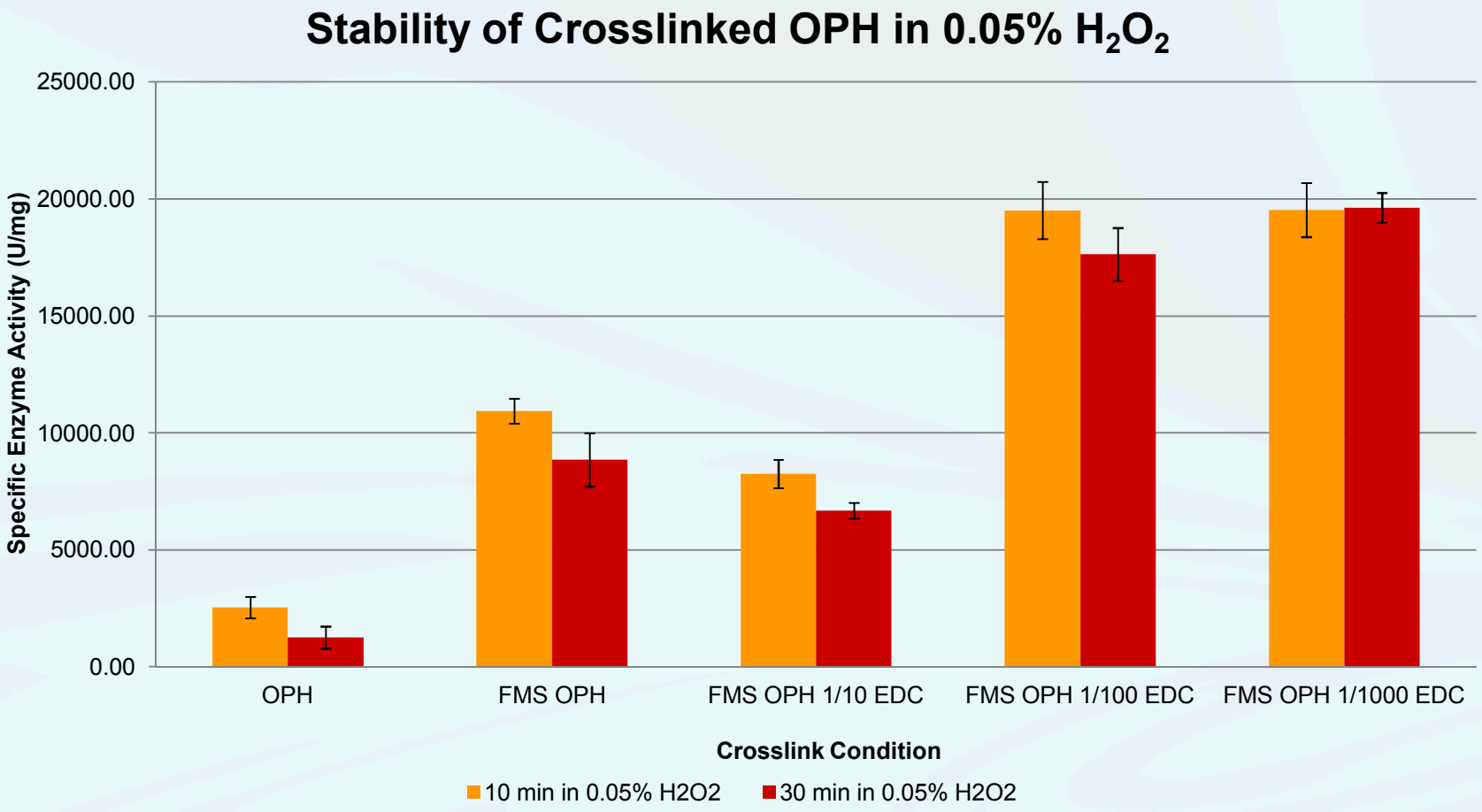
Crosslinking of OPH to FMS can improve specific activity.
Dependence of crosslinker concentration to enhance specific activity.

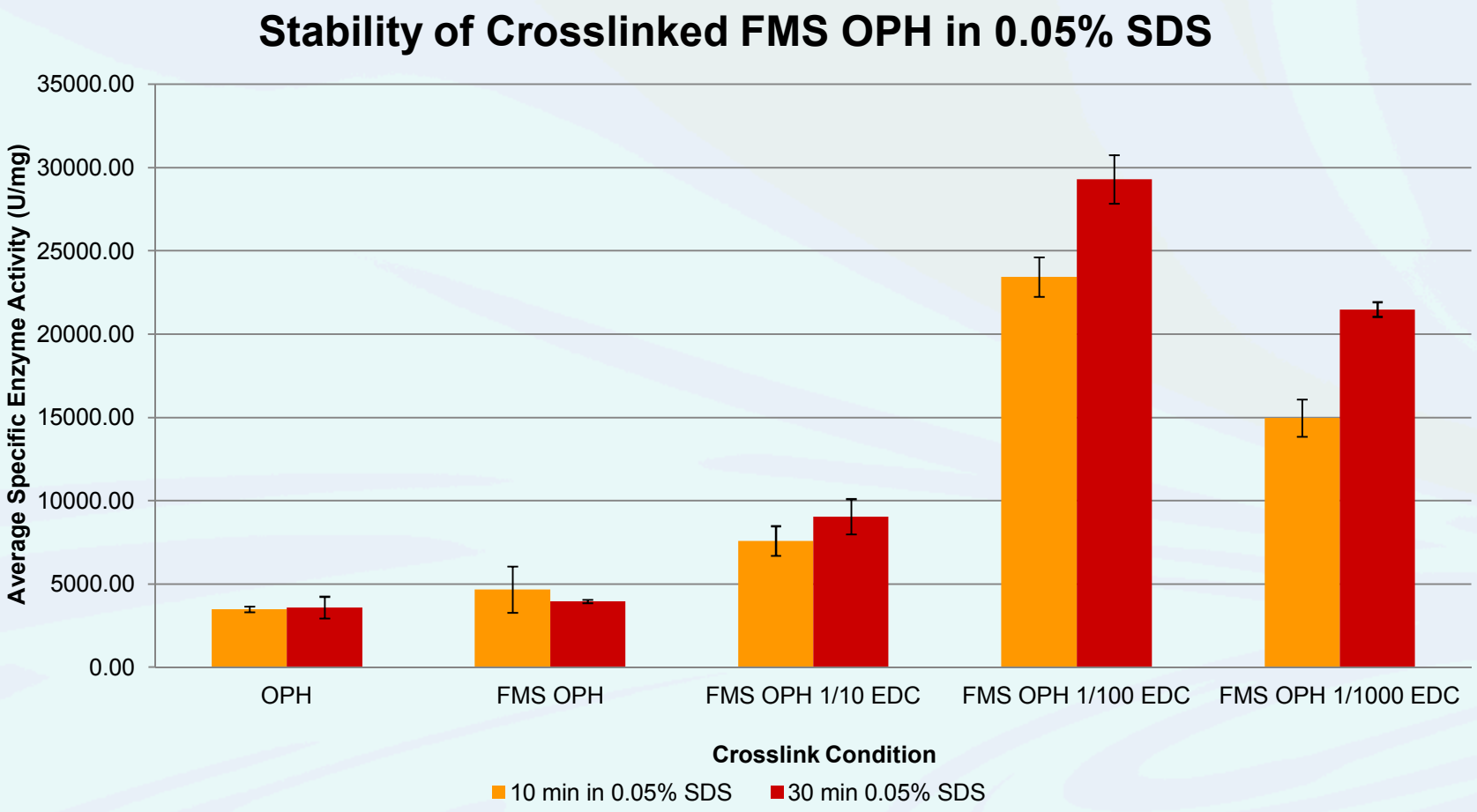
(Not shown: SH-SH maleimide bifunctional crosslinking reagent inhibited activity using FMS with SH groups.)

Tested Stability of Crosslinked OPH in FMS

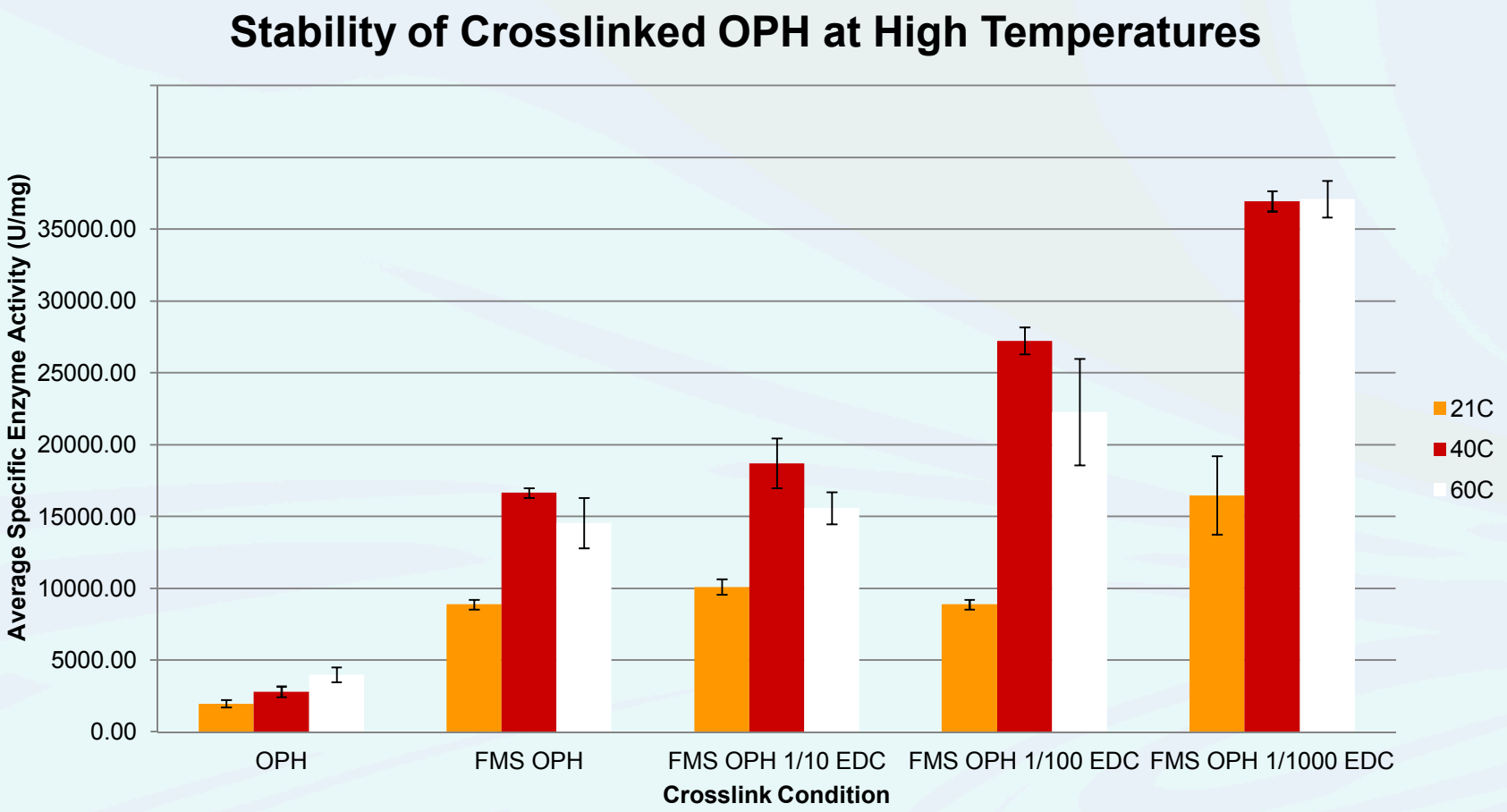
Purpose: Does crosslinking OPH to FMS improve overall enzyme stability?



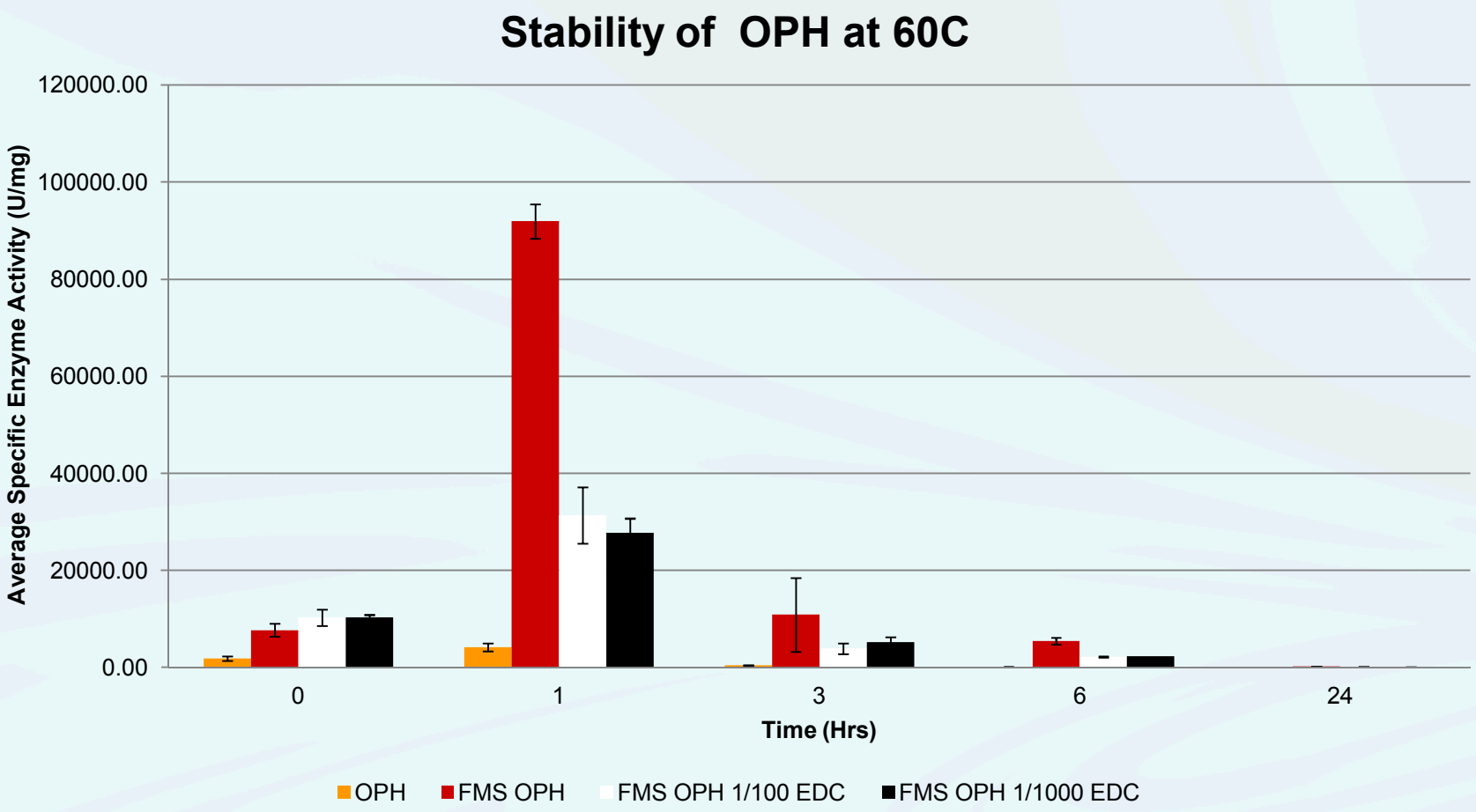

Stability of Crosslinked OPH in 0.05% H_2O_2
25000.00
20000.00
15000.00
10000.00
5000.00
0.00
Specific Enzyme Activity (U/mg)
OPH
FMS OPH
FMS OPH 1/10 EDC
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC
Crosslink Condition
10 min in 0.05% H2O2
30 min in 0.05% H2O2


Stability of Crosslinked FMS OPH in 0.05% SDS
Average Specific Enzyme Activity (U/mg)
35000.00
30000.00
25000.00
20000.00
15000.00
10000.00
5000.00
0.00
OPH
FMS OPH
FMS OPH 1/10 EDC
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC
Crosslink Condition
10 min in 0.05% SDS
30 min 0.05% SDS

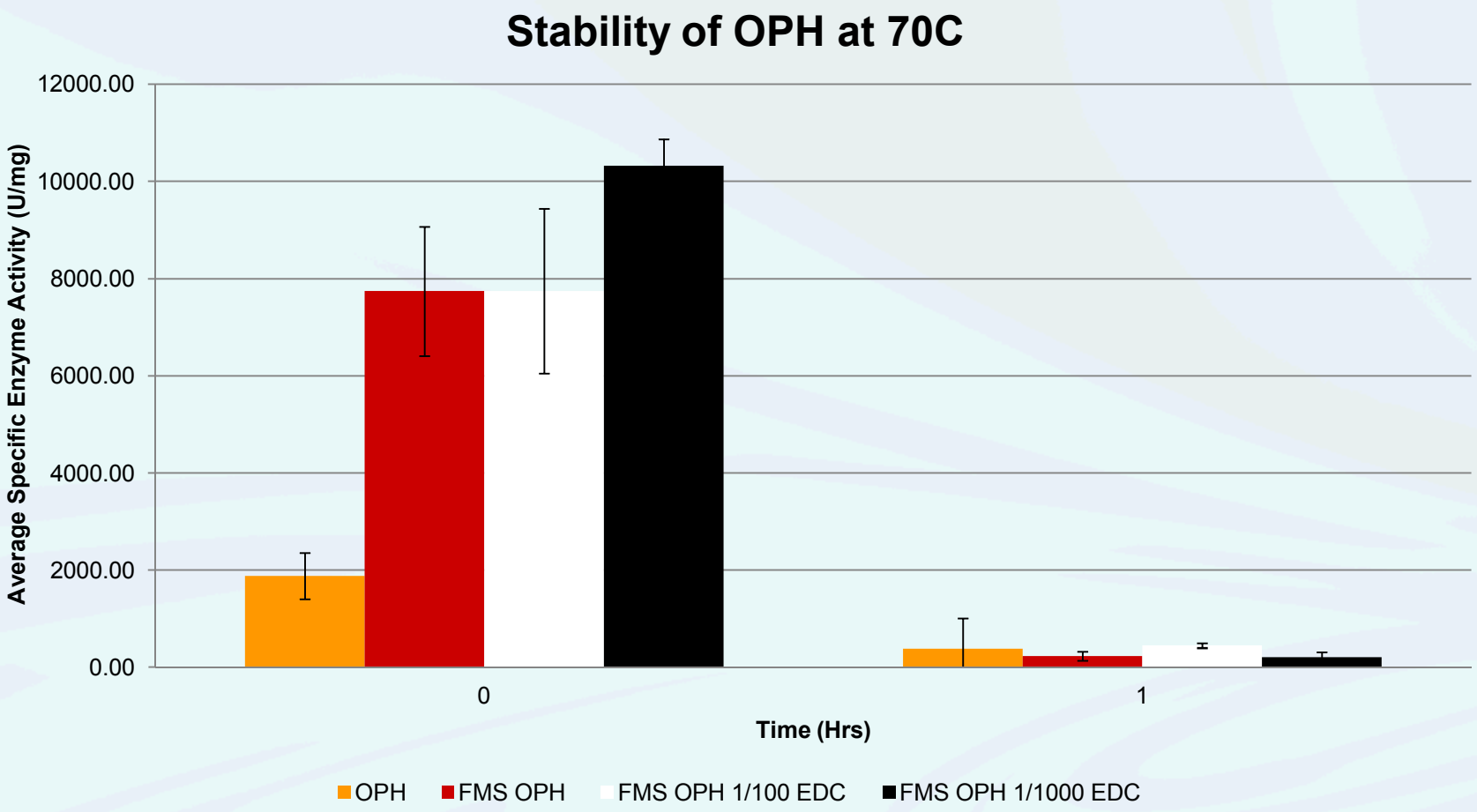
Results

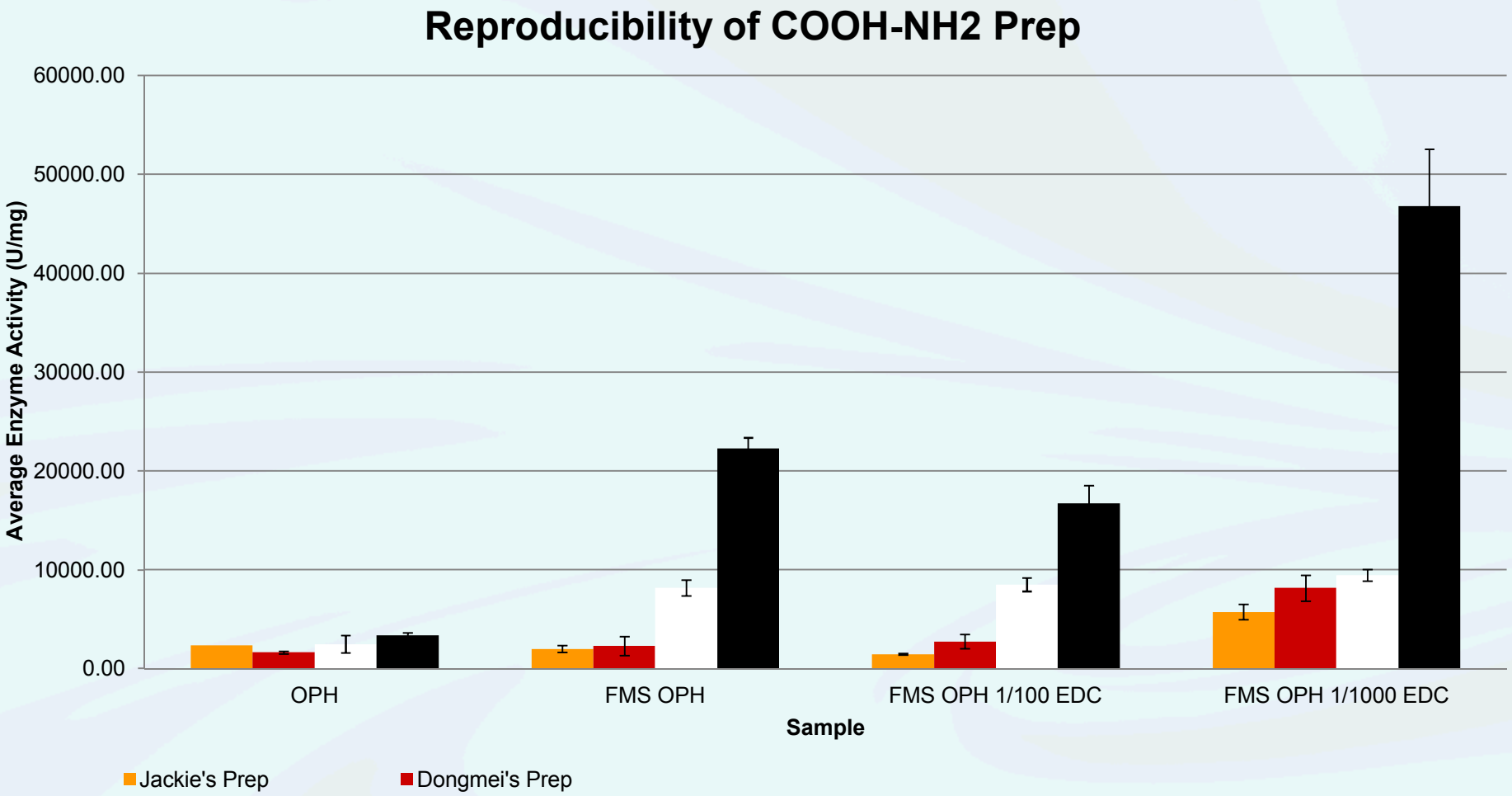

Stability of Crosslinked OPH at High Temperatures
Average Specific Enzyme Activity (U/mg)
0.00
5000.00
10000.00
15000.00
20000.00
25000.00
30000.00
35000.00
OPH
FMS OPH
FMS OPH 1/10 EDC
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC
Crosslink Condition
21C
40C
60C

Results


Stability of OPH at 60C
120000.00
100000.00
80000.00
60000.00
40000.00
20000.00
0.00
Average Specific Enzyme Activity (U/mg)
0
1
3
6
24
Time (Hrs)
OPH
FMS OPH
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC

Results

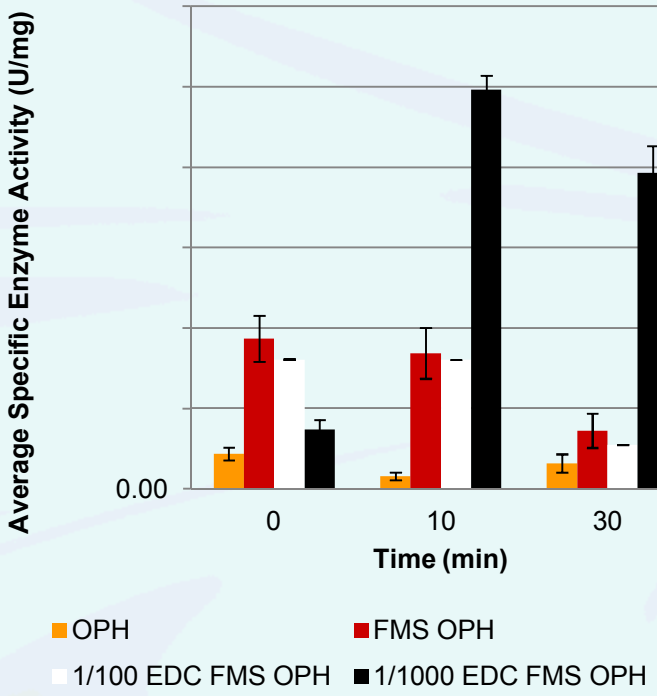

Stability of OPH at 70C
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
Average Specific Enzyme Activity (U/mg)
0
1
Time (Hrs)
OPH
FMS OPH
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC


Reproducibility of COOH-NH2 Prep
60000.00
50000.00
40000.00
30000.00
20000.00
10000.00
0.00
Average Enzyme Activity (U/mg)
OPH
FMS OPH
FMS OPH 1/100 EDC
FMS OPH 1/1000 EDC
Sample
Jackie's Prep
Dongmei's Prep

Conclusions

- Crosslinker significantly improves OPH stability in SuperSoap, SDS and H2O2.
- Crosslinker enhances OPH stability over short time at high temperature.
- Same basic results with Doug Cerasoli's OPH sample.

Stability in 1/15 SuperSoap (manufacturer's rec)

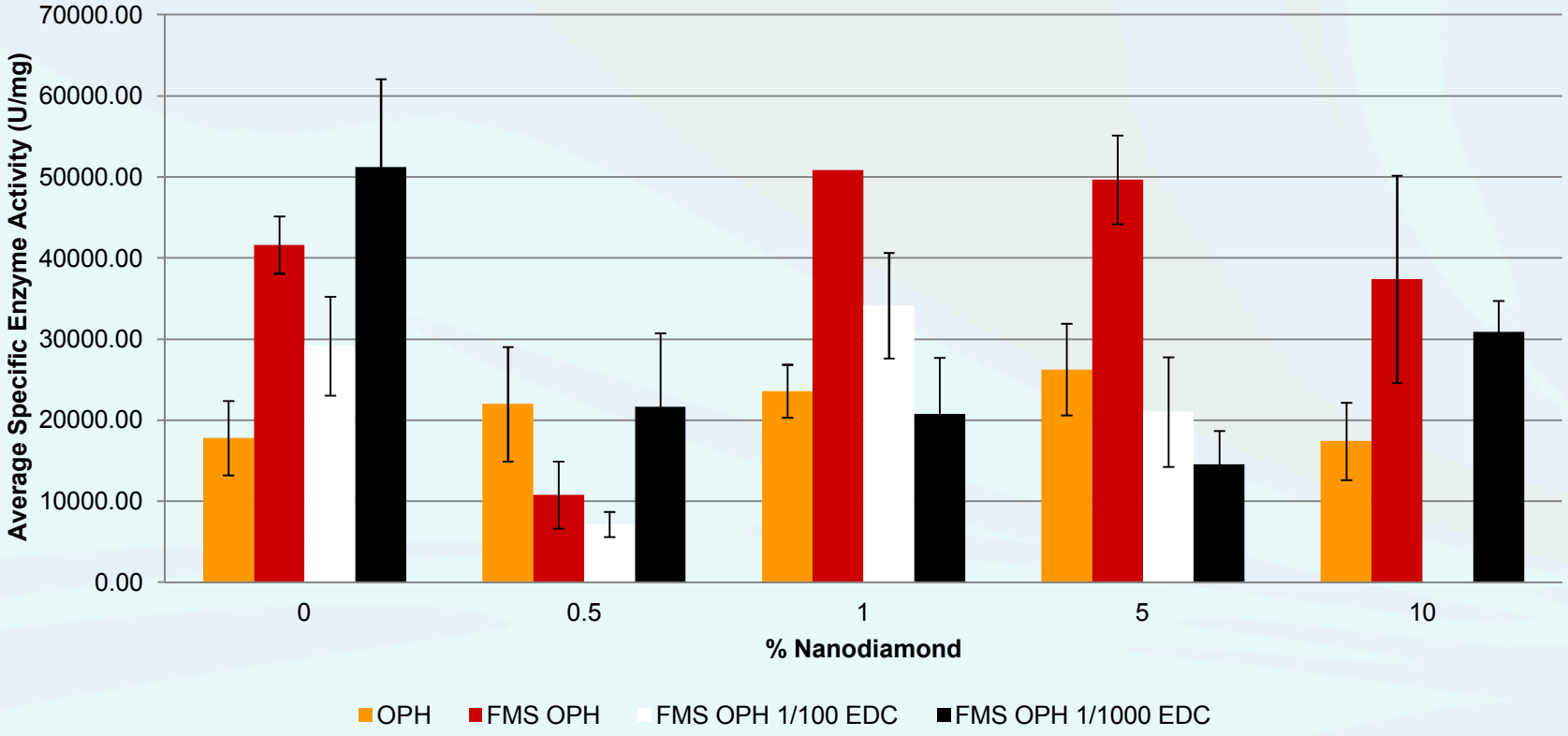


Will Crowding Reagents Improve Stability?

Nanodiamonds or Carbon Nanospheres

- **4-6 nm Carbodeon Superhard Nanomaterials.**
- **Nanodiamond Specifications:**
  - Content in solid phase ~ 97 wt.%
  - Oxidizable carbon content in solid phase ≤ 2.5%
  - Metallic incombustible impurity content in solid phase ≤ 1.2 wt.%
  - Bulk Density ~ 0.5 gm/cm^3
  - Pycnometric density ~ 3.1-3.2 gm/cm^3
  - Specific surface area 330 gm/cm^3
  - Constant of crystal lattice 0.3573 ± 0.0005 nm
  - Graphitization in vacuum, starting at ~ 1100^{0}C
  - Oxidation in air, starting at ~ 450^{0}C

Stability of USAMRICD OPH in Suspension with Nanodiamonds

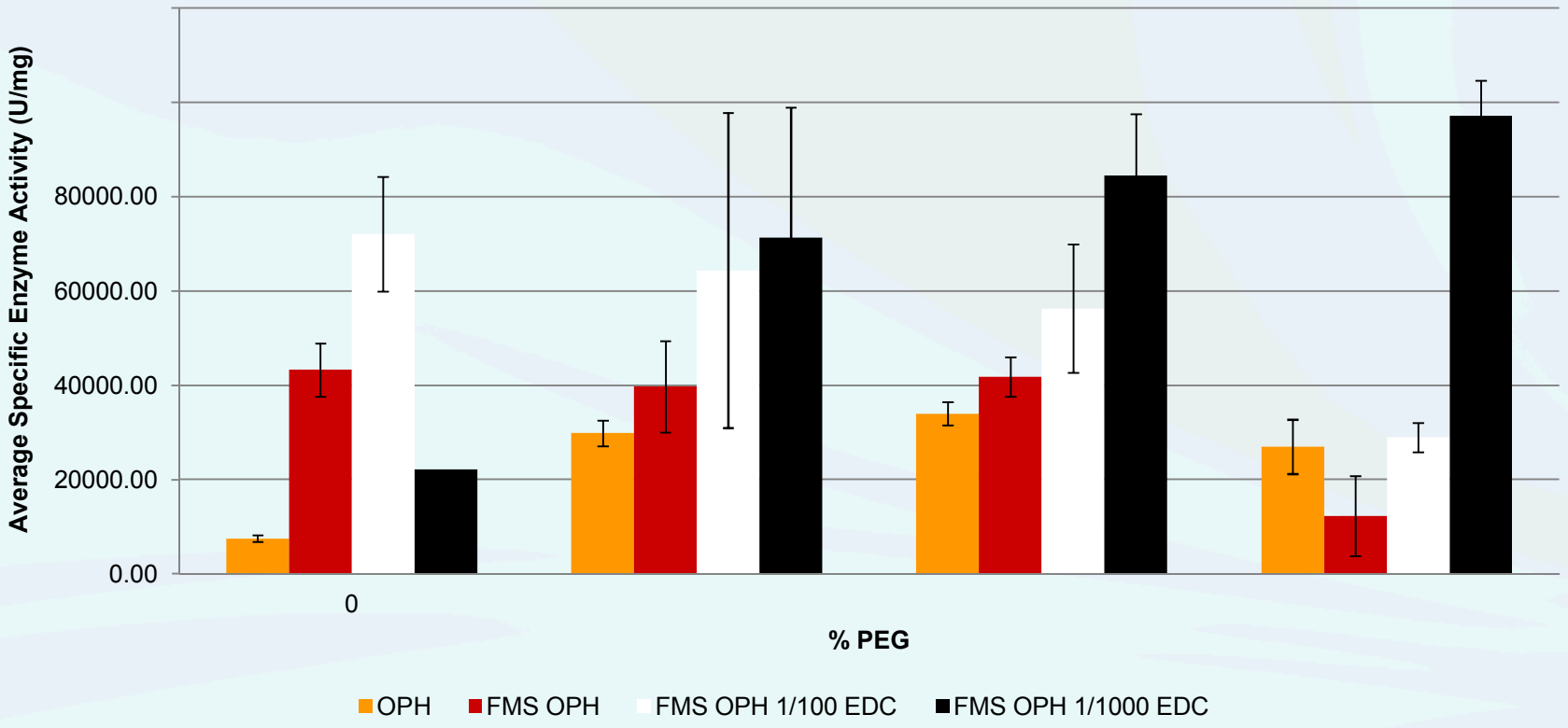


- Nanodiamonds have somewhat inhibitory effect on specific activity.
- There was a slight improvement with FMS OPH.

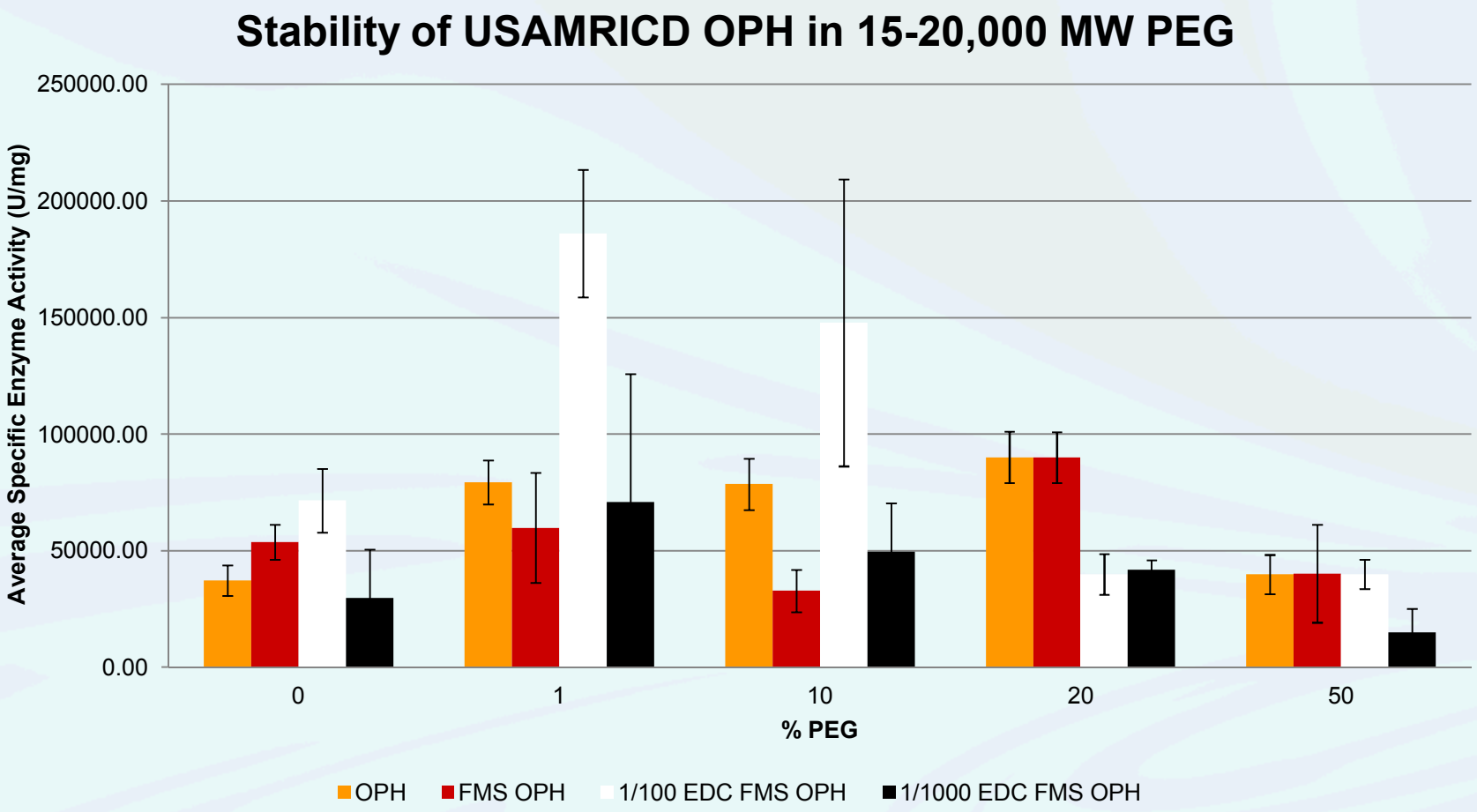
Polyethylene Glycol (PEG) Protocol

- Prepared different concentrations of PEG (0, 1, 10, 20%) and different MW PEGs (15-20,000, 400, 4,000, & 10,000) in 100 mM CHES (pH 9) with 50 uM zinc acetate.
- Made 1/500 dilutions of OPH, FMS OPH, FMS OPH 1/100 EDC, and 1/1000 FMS OPH in each of the PEG solutions.
- Vortexed briefly and measured enzyme specific activity

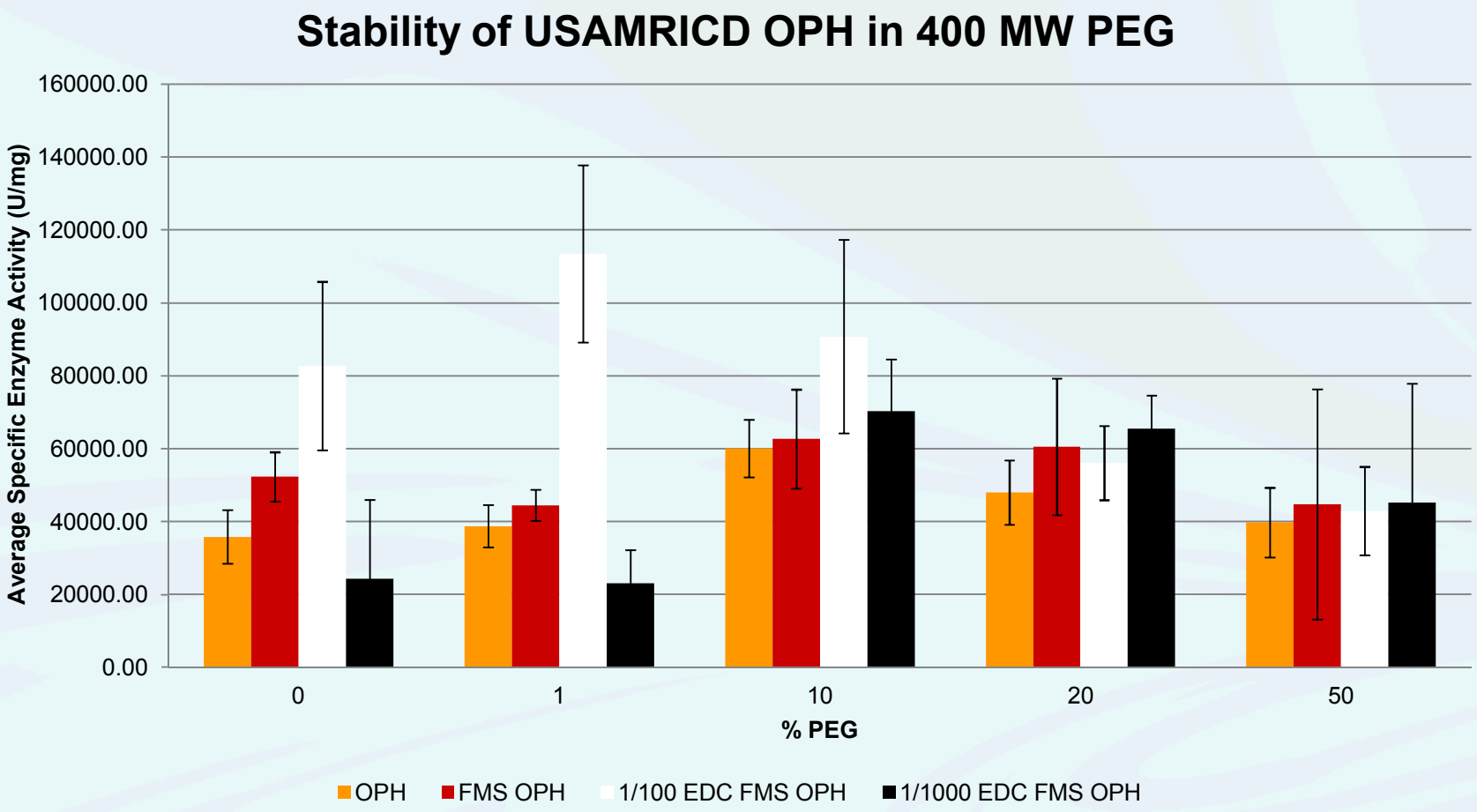
Stability of USAMRICD OPH in Suspension with MW 10,000 PEG



Results


Stability of USAMRICD OPH in 15-20,000 MW PEG
Average Specific Enzyme Activity (U/mg)
250000.00
200000.00
150000.00
100000.00
50000.00
0.00
0
1
10
20
50
% PEG
OPH
FMS OPH
1/100 EDC FMS OPH
1/1000 EDC FMS OPH

Results


Stability of USAMRICD OPH in 400 MW PEG
Average Specific Enzyme Activity (U/mg)
160000.00
140000.00
120000.00
100000.00
80000.00
60000.00
40000.00
20000.00
0.00
0
1
10
20
50
% PEG
OPH
FMS OPH
1/100 EDC FMS OPH
1/1000 EDC FMS OPH

Conclusions

- Glycerol does not have much of an effect (not shown).
- Nanodiamonds or carbon nanospheres inhibitory for crosslinked protein but ~stimulatory for uncrosslinked.
- 15-20,000 MW PEG shows some promise in further improving immobilized enzyme activity.

Controlling OPH Conformation During Immobilization Crosslinking

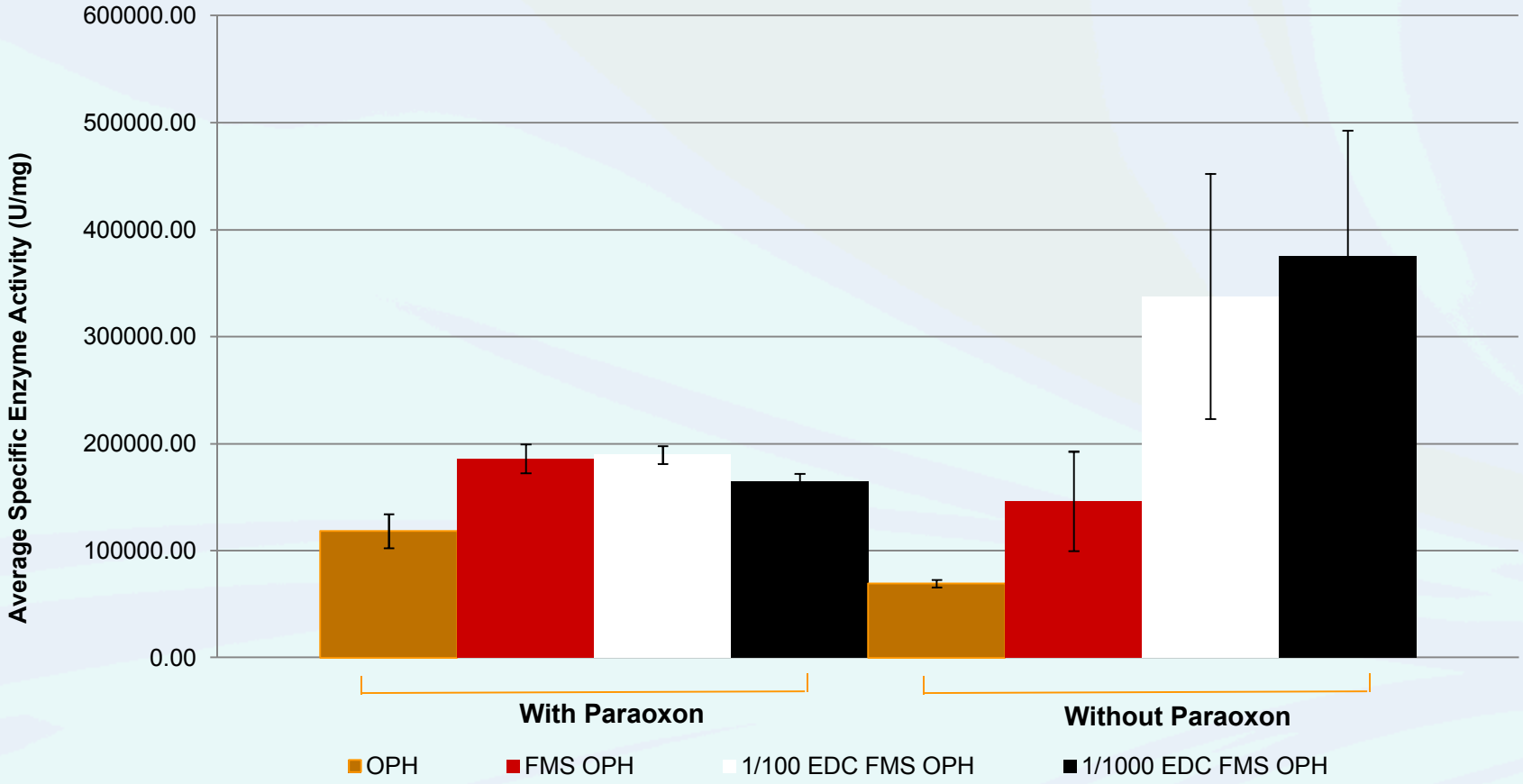
Purpose: Assess whether immobilization in the presence of Paraoxon enhances specific activity.

Hypothesis arose because presence of excess paraoxon during temperature stability measurements led to greater thermal stability.

Procedure

- Prepared COOH-NH2 crosslinking reaction as described previously using the USAMRICD OPH.
- Prepared a second batch following protocol except added paraoxon to OPH before crosslinking; *i.e.,* add OPH + paraoxon to activated FMS.

Efficiency of OPH Specific Activity when Immobilized with FMS in Paraoxon



Conclusions

- **Paraoxon added to OPH before crosslinking reaction to FMS can be inhibitory under initial conditions.**
- **Is there is an optimum concentration that will not be inhibited by Paraoxon?**

  and demonstrate benefits of maintaining active-site conformation during crosslinking?

- **Overall, OPH alone:**

  < OPH FMS

  < " " + Zn (or Co)

  < " " " + crosslinking

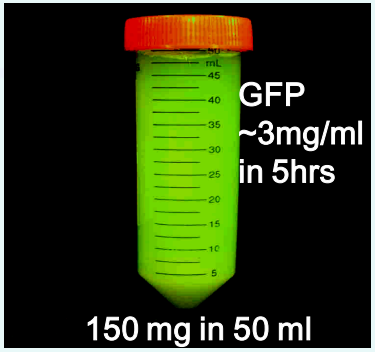
  < " " " " + excess substrate

Part II: Cell-free translation of rational OPH mutants with higher thermal stabilities and activities

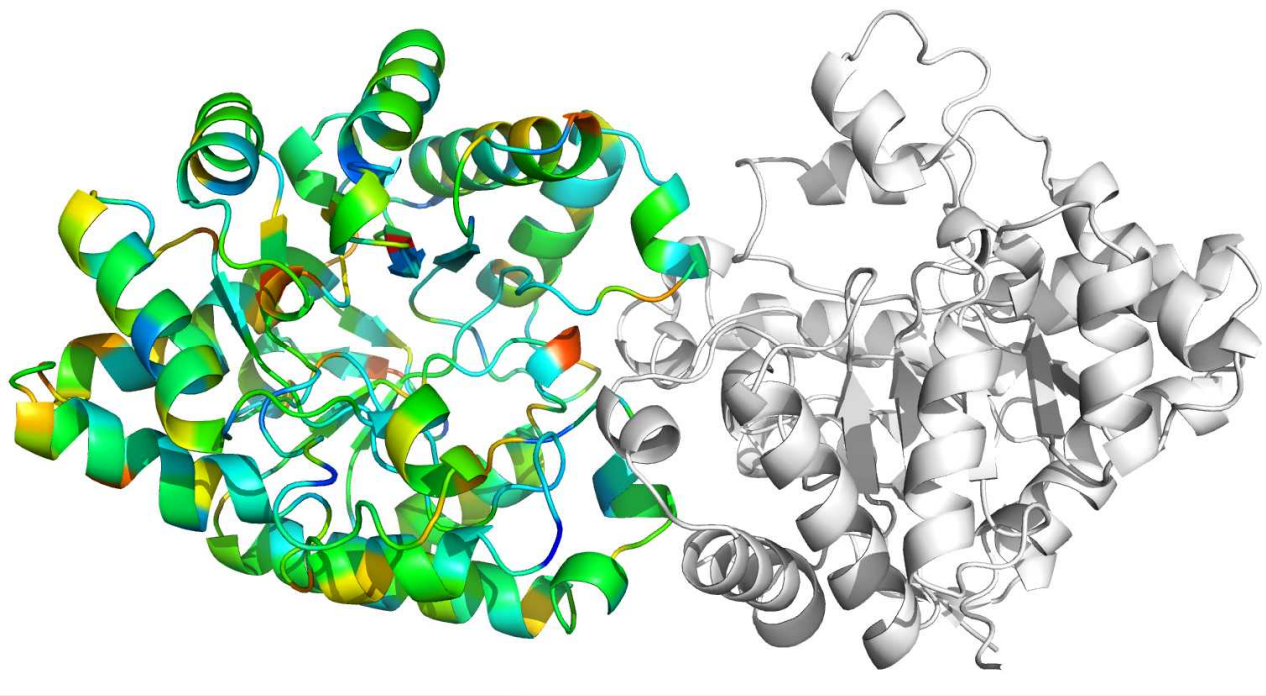
Advantages of Cell-free Approach:

- Faster, less expensive than traditional cellular-based methods
- Proteins can be made directly from PCR templates, eliminating costly, time-consuming steps for plasmid cloning
- Multiple conditions can be screened simultaneously
- Multiple proteins can be expressed simultaneously in one reaction to facilitate production of active protein complexes
- Proteins can be characterized directly from the translation reactions.

CFS GenDecoder robot capable of producing 384 proteins/day.



Putative hotspots for stabilizing mutations

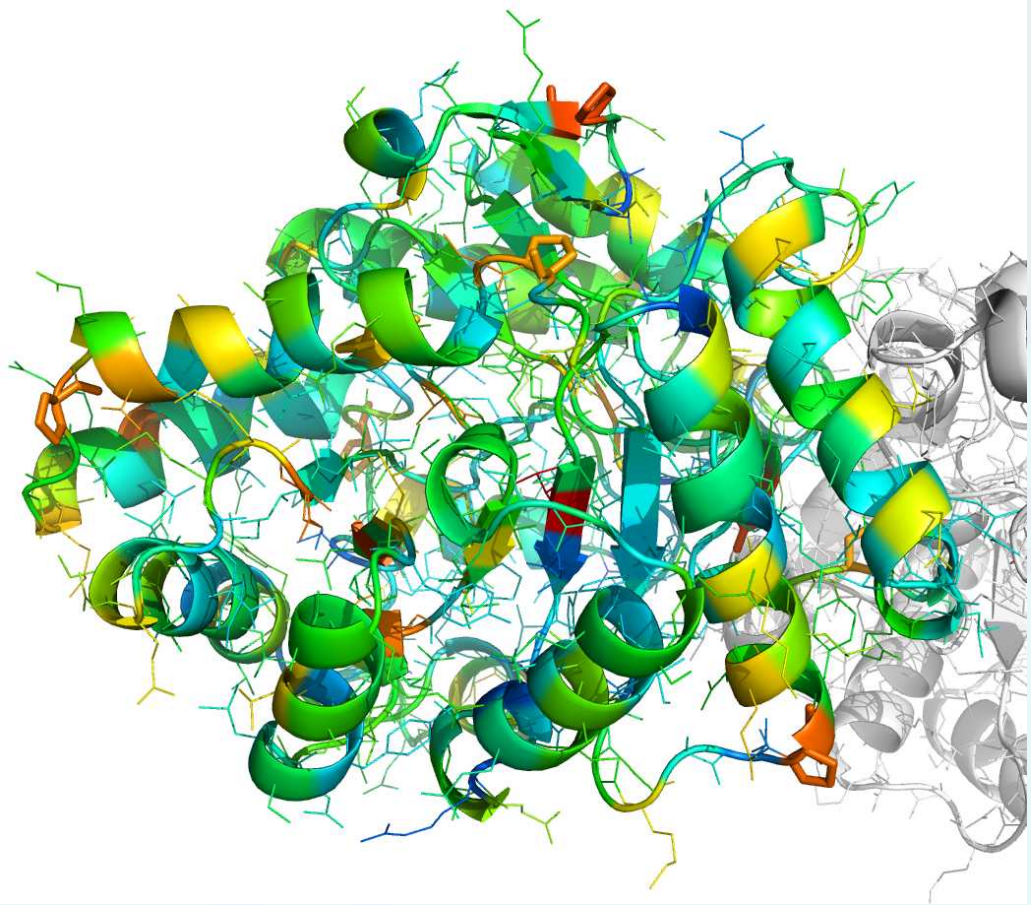


OPH dimer (PDB: 1DPM)

1. **Residues are color-coded (from blue to red) according to the ΔΔG upon mutations.**
2. **Red corresponds to high stabilization effect.**
3. **The metal-coordinating and ligand-binding residues are not selected for mutation.**

✓**The majority of the positions does not have a strong stabilizing mutations.**
✓**There are a few hotspots that can be mutated to increase the stability.**

Hotspots: Proline?



Many conserved prolines, which are picked up by Medusa/Eris as possible stabilizing hotspots.

These proline residues function as the capping of secondary structures, which might not be amenable for mutations without affecting the structure and folding dynamics.

Predicted candidates

Residue	Mutation	Predicted ddG (kcal/mol)
T54	T53Met	-5.0 (Z = -2.0)*
D105	D105Thr	-6.7 (Z = -2.7)
T199	T199Ile	-8.1 (Z = -3.3)
C227	C227Met	-3.8 (Z = -1.5)
G251	G251Leu	-6.7 (Z = -2.7)

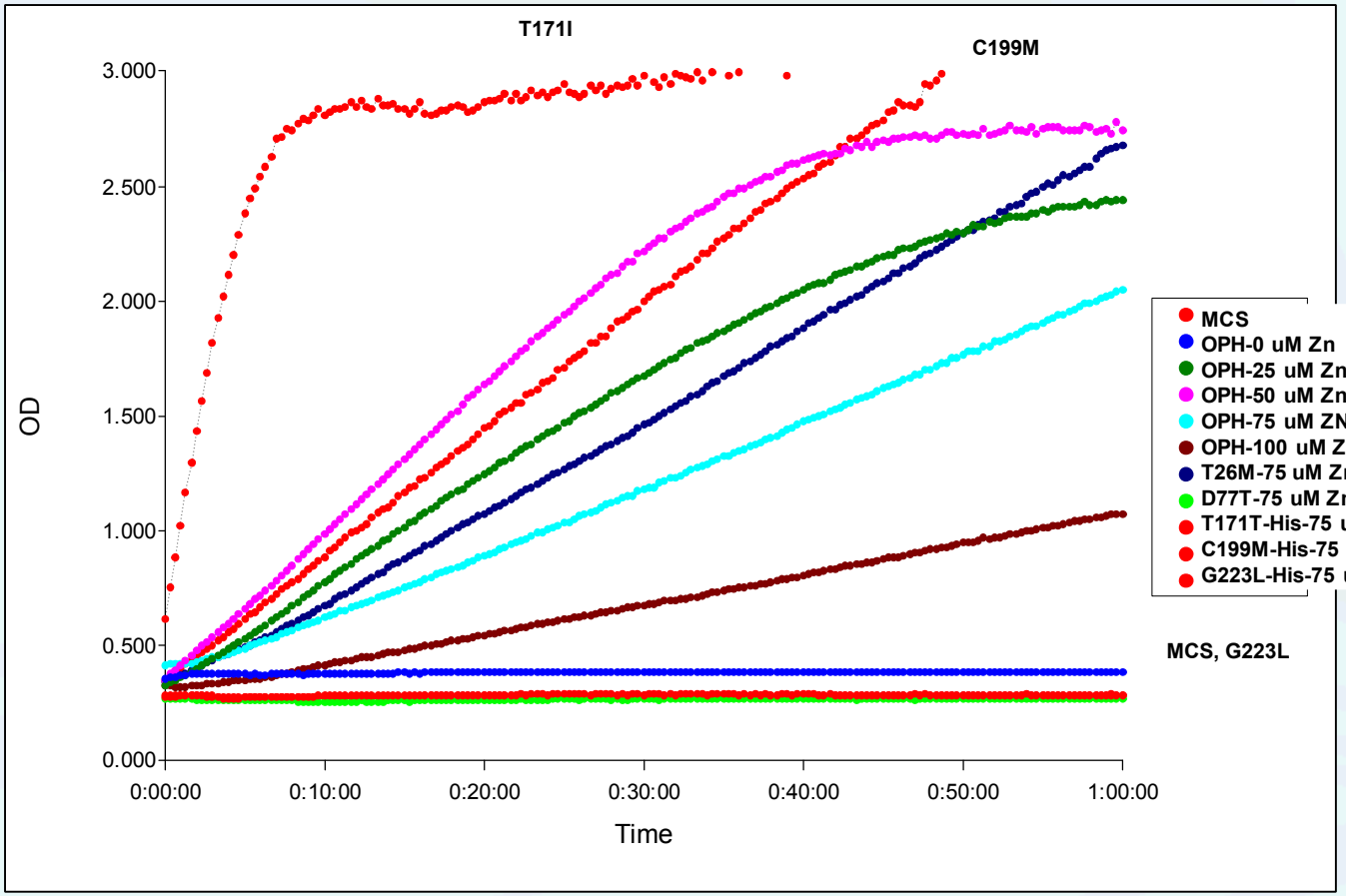
***Z-score is estimated by 100 rounds simulation for both wild type, and mutant**

Sandia National Laboratories

Sequence alignment of OPH wild-type and mutants

Sandia National Laboratories

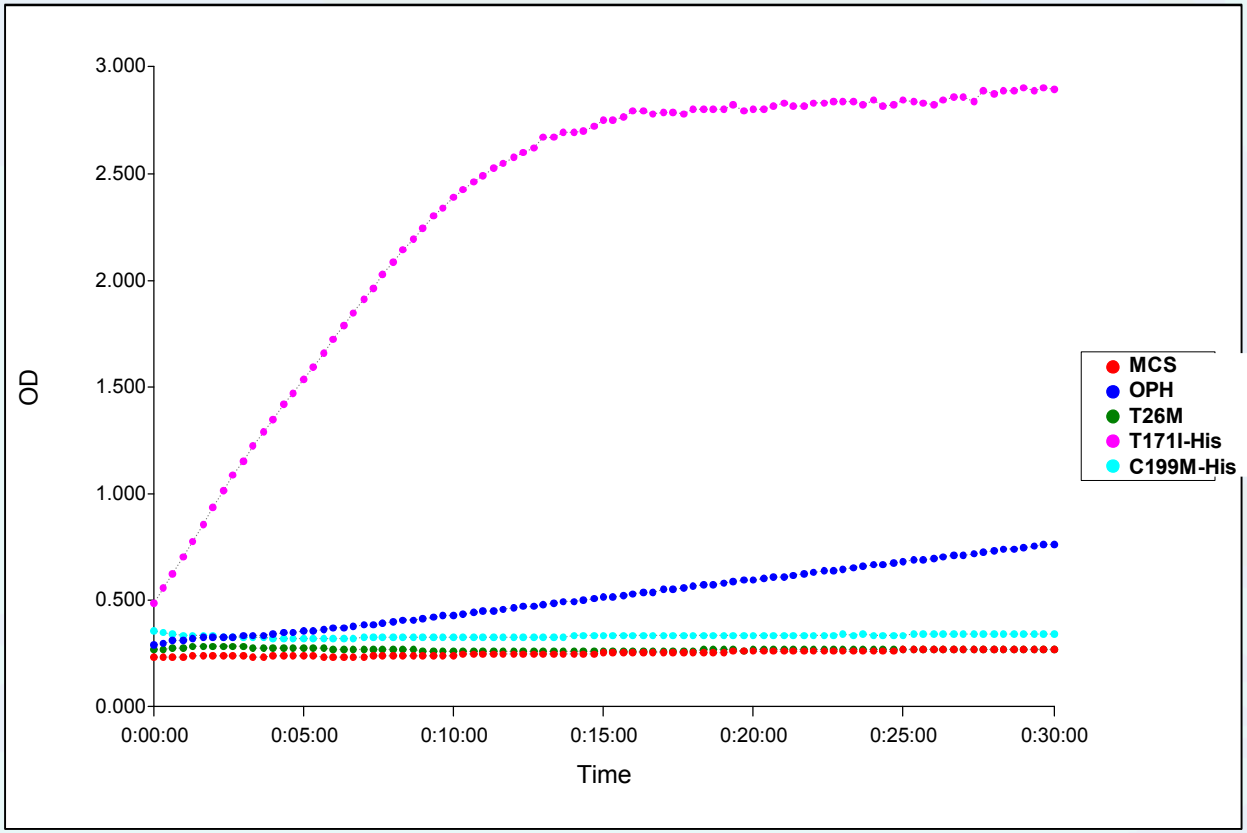
Different [$Zn(OAc)_2$] used in CFS translation of WT OPH; 75 uM used for OPH mutant. 2 ul of CFS reaction was used for one activity assay.



Assay condition: 2 ul of CFS OPH or mutant was added to 200 ul of CHES buffer containing same conc. of Zn as in it's CFS translation, activity was determined: 5 s mix, O.D. 405nm was read every 20 s for 1h.

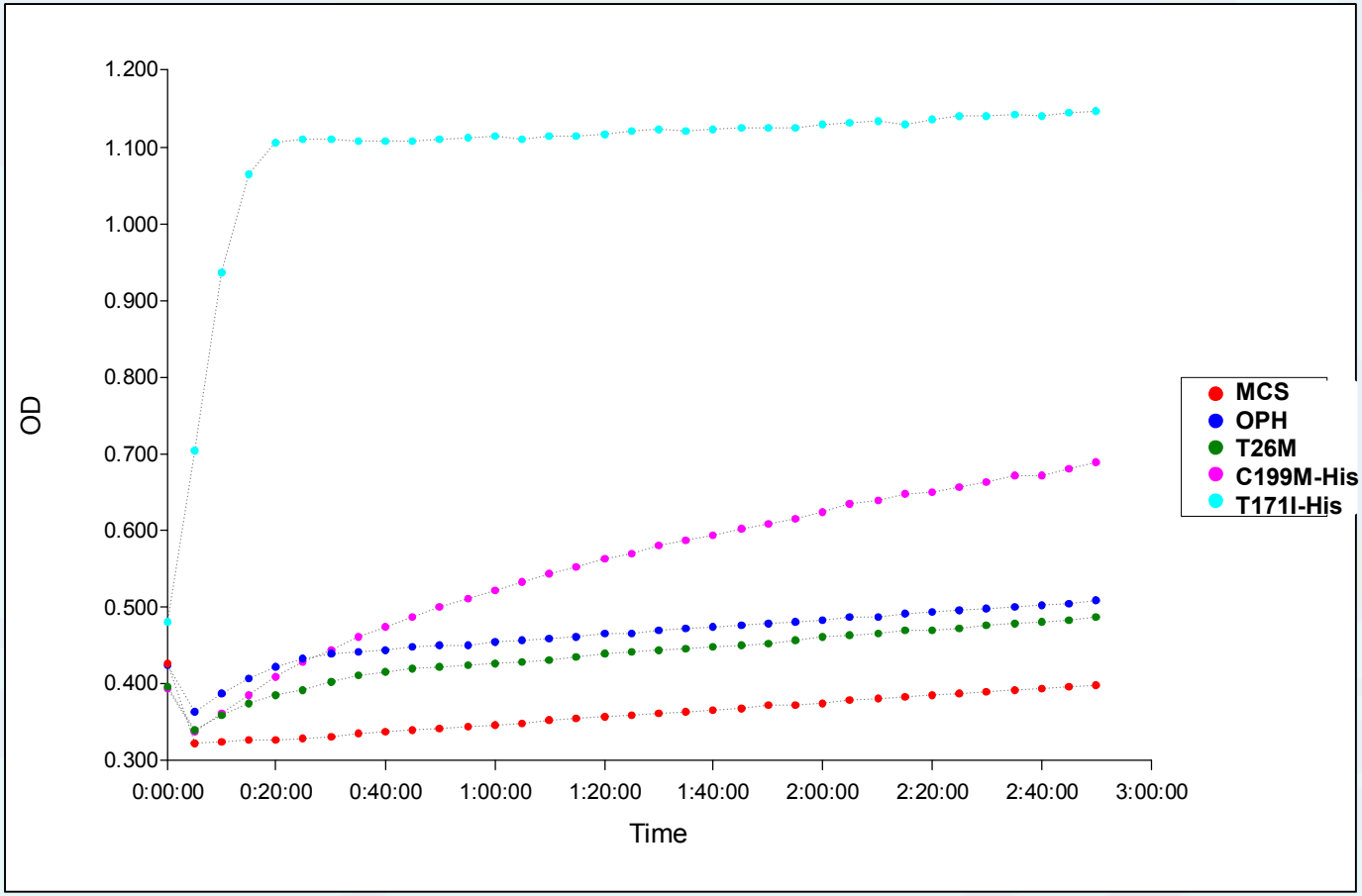
T171I has much higher activity than WT OPH, T26M and C199M has similar activity as WT OPH. D77T and G223L are not active.

Large scale CFS reaction, 50 uM $Zn(OAc)_2$ during translation.

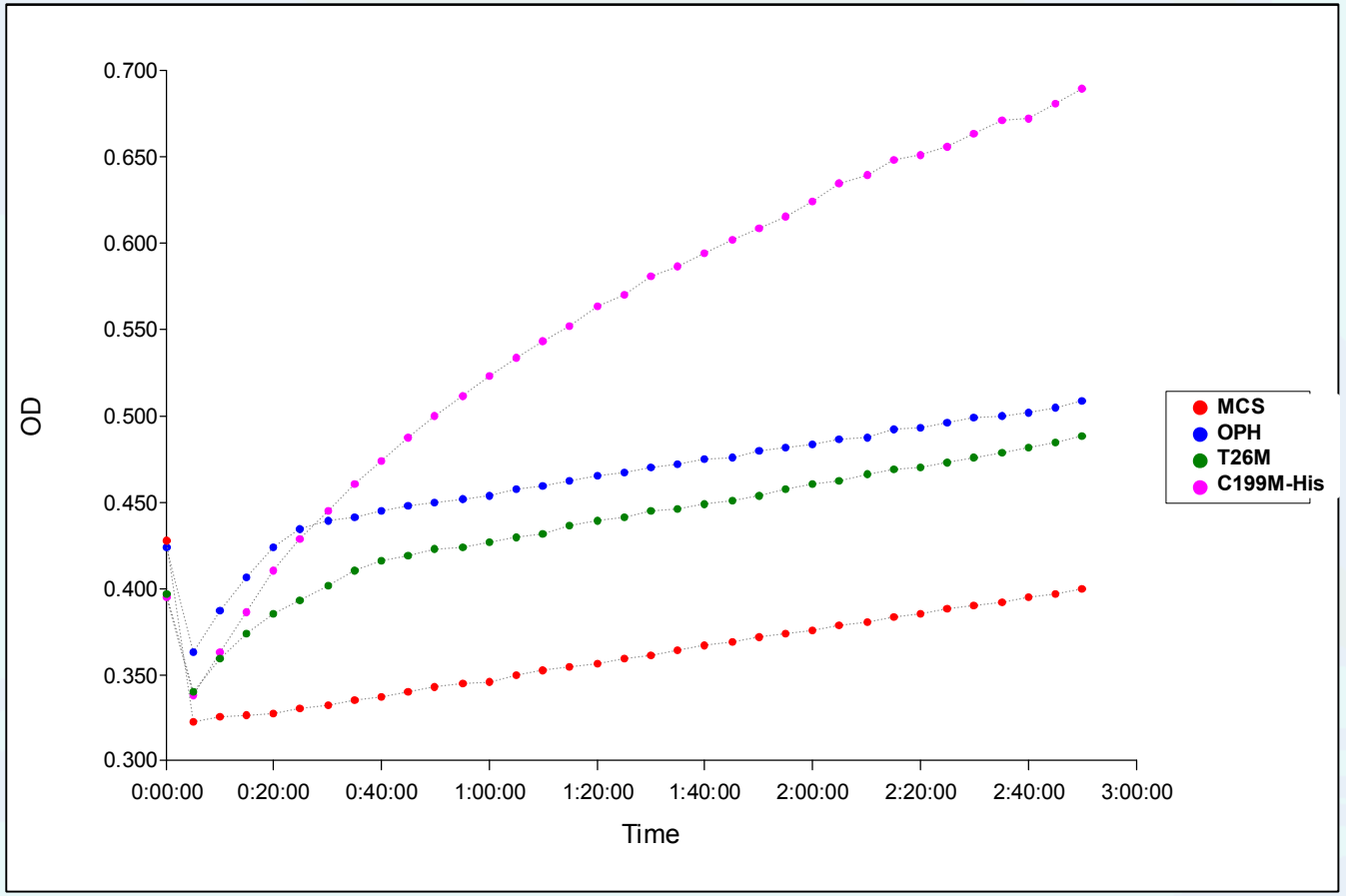


Assay condition: 2 ul of CFS OPH was added to 200 ul of CHES buffer containing 50 uM of Zn(OAc)2; activity: 5 s mix, O.D. 405 nm was read every 20 s for 30 min.

Use optically-clear adhesive film for long temp expts. to prevent condensation volume losses and damage to plate reader.



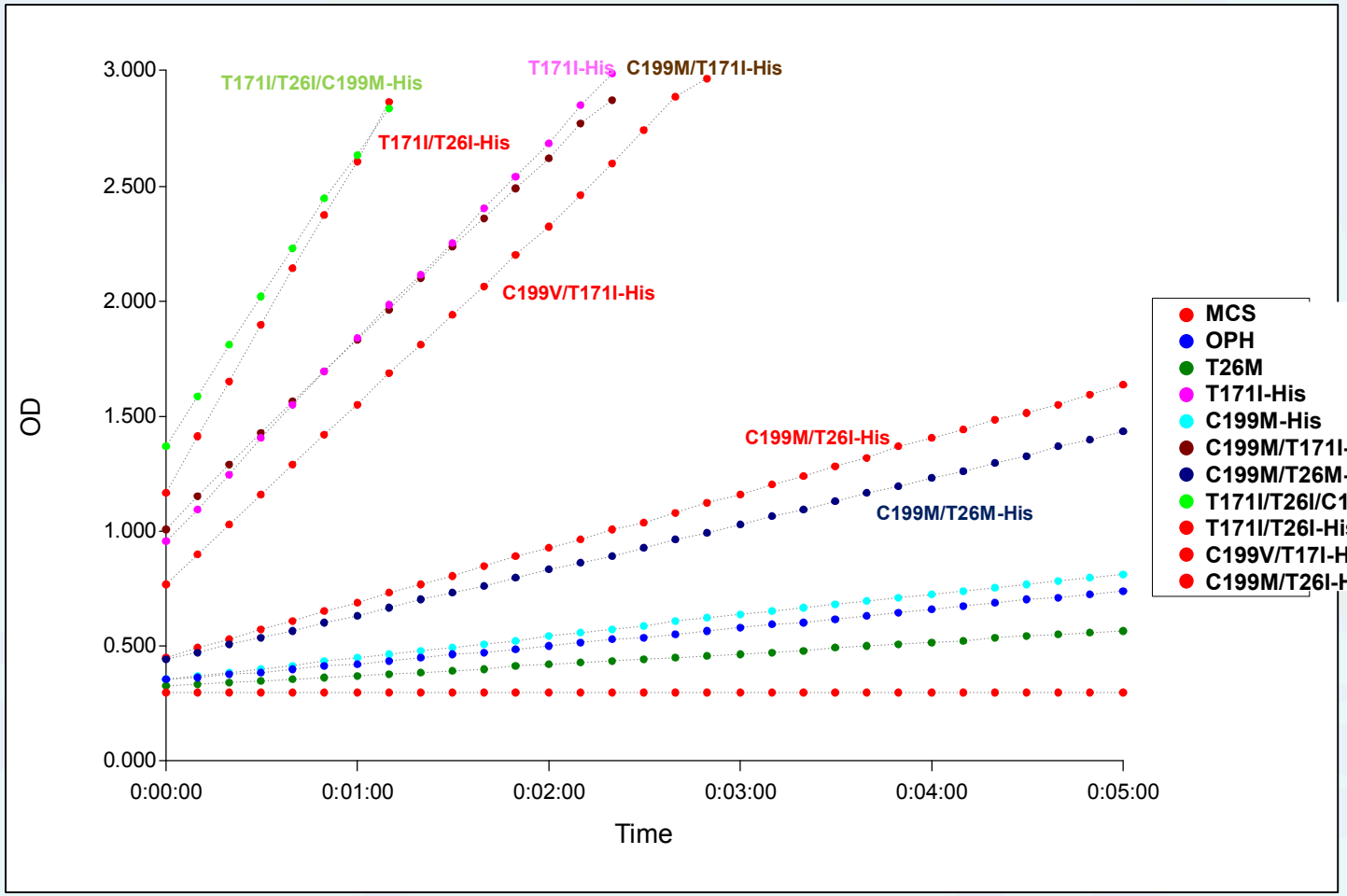
Assay condition: CFS OPH and mutants were diluted in CHES buffer at 1:20 ratio, then add 2ul into 200 ul pre-heated CHES buffer containing Zn. Eon was pre-heated to 60C as well. Paraoxon was added to a final conc of 1mM. Activity was determined: at 60C, 5 s mix, O.D. 405nm was read every 5 min for 3 h.



Same figure except T171I-His is not included.

Are beneficial effects of mutations additive?

	Expressed	No Expression
1. Single mutants:	T26M	T26M-His
	D77T	D77T-His
	T171I-His	T171I
	C199M-His	C199M
	G223L-His	G223L
2. Double mutants:	C199M/T171I-His	
	C199M/T26M-His	
		T171I/T26M-His
3. Triple mutant:		T171I/T26M/C199M-His


3.000
2.500
2.000
1.500
1.000
0.500
0.000
OD
0:00:00
0:01:00
0:02:00
0:03:00
0:04:00
0:05:00
Time
T171I/T26I/C199M-His
T171I-His
C199M/T171I-His
T171I/T26I-His
C199V/T171I-His
C199M/T26I-His
C199M/T26M-His
MCS
OPH
T26M
T171I-His
C199M-His
C199M/T171I-His
C199M/T26M-His
T171I/T26I/C199M-His
T171I/T26I-His
C199V/T17I-His
C199M/T26I-His

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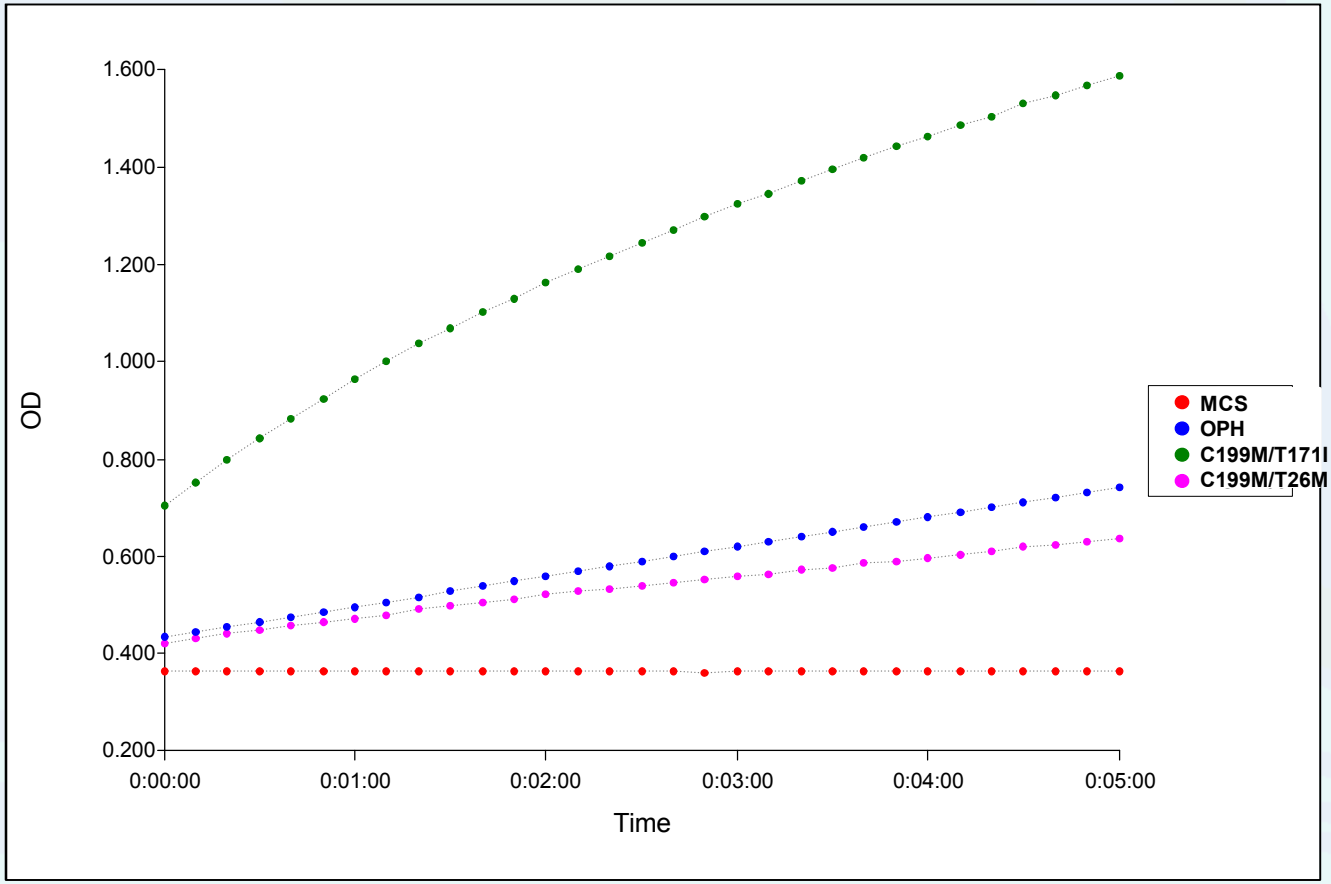
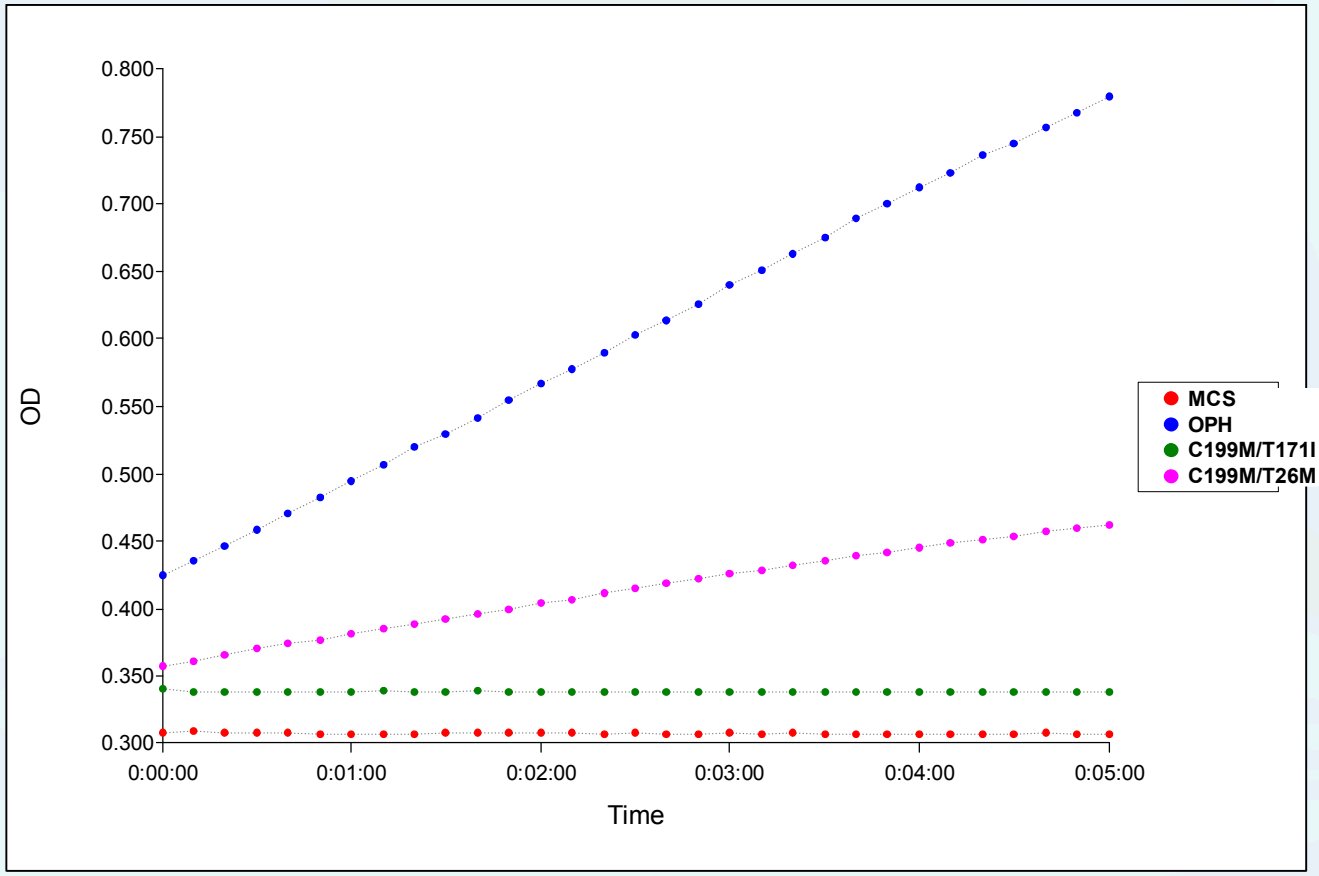
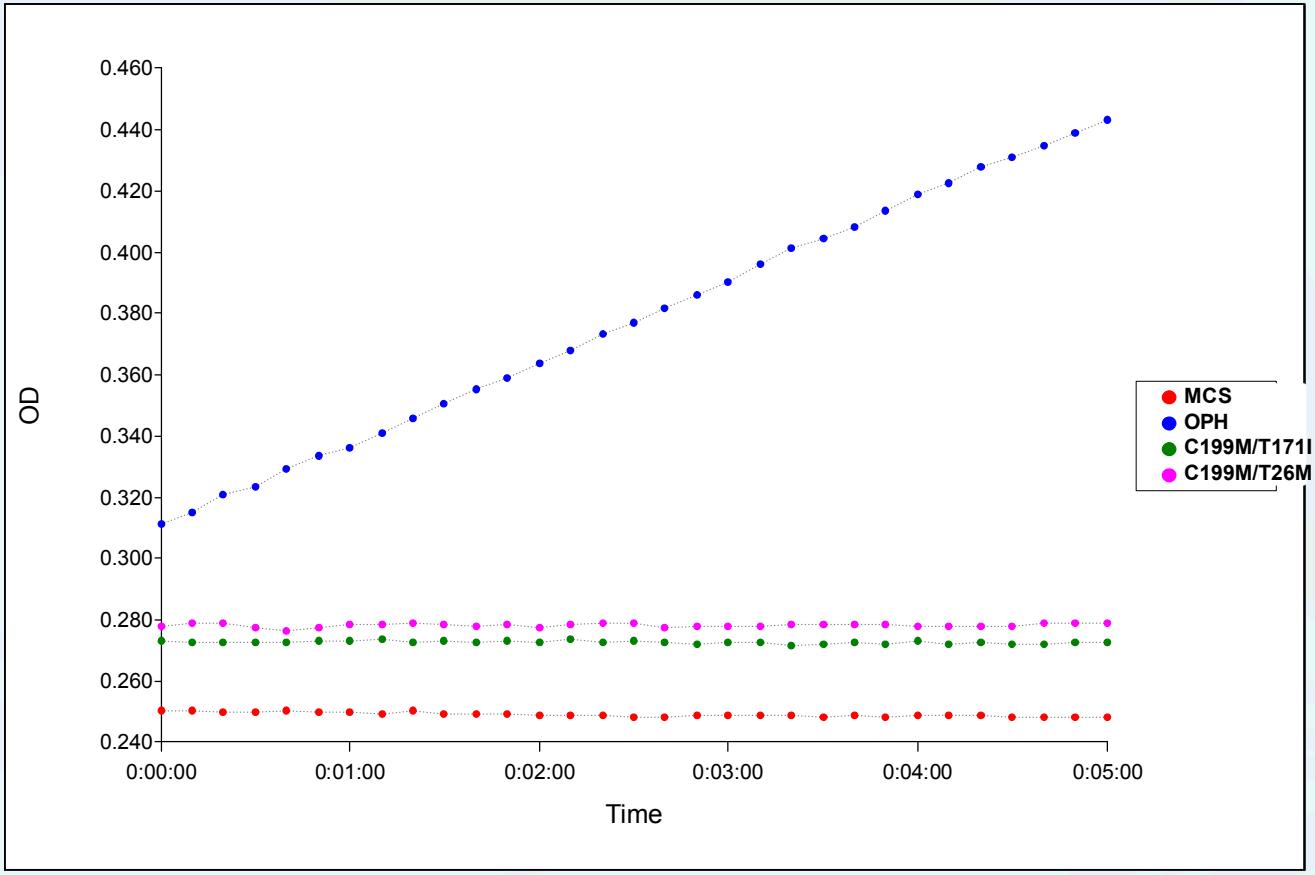
Time course of OPS effect on C199M/T171I and C199M/T26M activities



At time 0 min, C199M/T171I is more active than WT OPH, but the activity is not as high as T171I single mutation. C199M/T26M is slightly less active that WT OPH by a tiny bit.



At time 10 min, C199M/T171I lost activity. C199M/T26M is still active but with a much lower activity than WT OPH.



At time 30 min, C199M/T171I and C199M/T26M both lost activity. WT OPH remains active.

New mutants: T171I/T26I/C199M-His
T171I/T26I-His
C199V/T171I-His
C199M/T26I-His

T26I works much better than T26M. Usage of T26I instead of T26M in double-mutation combination increased overall activity.

Double mutants (C199M/T171I-His and C199M/T26M-His) don't decrease activity *vs.* their single mutants. All samples made using same master mix and activities were measured simultaneously.

Confirmed sequences of Saumil's mutants listed below. They are all correct.

R13E, R13E-His,
R13E/R63E, R13E/R63E-His
R13E/R63E/D93K, R13E/R63E/D93K-His
R13E/R63E/D93K/K266E, R13E/R63E/D93K/K266E-His

H95K, H95K-His
H95K/R136E, H95K/R136E-His
H95K/R136E/K311E, H95K/R136E/K311E-His
H95K/R136E/K311E/R328E, H95K/R136E/K311E/R328E-His

Amino acid sequence alignment of OPH5.1 vs. wt OPH.First five amino acids from N-term are replaced by his-tag in OPH 5.1.

```
OPH5.1      1  MGHHHHHHHH  10
WT OPH      1  M      SIGT   5

OPH5.1     11  GDRINTVRGPIEISKAGFTLTHEHICGSSAGFLRAWPEFFGSRKALAEKAVRGLREARAA  70
WT OPH      6  GDRINTVRGPITISEAGFTLTHEHICGSSAGFLRAWPEFFGSRKALAEKAVRGLRRARAA  65

OPH5.1     71  GVETIVDVSTFDIGRDVSLLAEVSRAADVHIVAATGLWFDPPLSMRLRSVEELTQFFLRE  130
WT OPH     66  GVRTIVDVSTFDIGRDVSLLAEVSRAADVHIVAATGLWFDPPLSMRLRSVEELTQFFLRE  125

OPH5.1    131  IQYGIEDTGIEAGIIKVATTGKATPFQELVLKAAARASLATGVPVTTHTAASQRDGEQQA  190
WT OPH    126  IQYGIEDTGIRAGIIKVATTGKATPFQELVLKAAARASLATGVPVTTHTAASQRDGEQQA  185

OPH5.1    191  AIFEEEGLSPSRVCIGHSDDTDDLSYLTALAARGYLIGLDHIPHSAIGLEDNASASALLG  250
WT OPH    186  AIFESEGLSPSRVCIGHSDDTDDLSYLTALAARGYLIGLDHIPHSAIGLEDNASASALLG  245

OPH5.1    251  IRSWQTRAKLIKALIDQGYKKQILVSNDWLFGFSSYVTNIMDVMDRVNPDGMAFIPKRVI  310
WT OPH    246  IRSWQTRALLIKALIDQGYMKQILVSNDWLFGFSSYVTNIMDVMDRVNPDGMAFIPLRVI  305

OPH5.1    311  PFLREEGVPQETLAGITVTNPAEFLSPTLRAS  342
WT OPH    306  PFLREKGVPQQTLAGITVTNPARFLSPTLRAS 337
```

Initial efforts to express Saumil's mutants failed due to presence of extra N-term amino acids and/or His-Tag. Recloned all mutants into our OPH gene and removed His-Tag. Stay tuned . . .

Conclusions and Future

Immobilizations → promote stability and activity

- Improvements: extra active-site metal & presence of substrate, crowding reagents, covalent
- Design materials for enzyme and enzyme for materials
- Design crosslinking sites into enzyme for max substrate delivery and product removal

Cell-free translation + modeling → promote stability and activity

- Improvements: thermal stability and activity
- Some mutations additive
- Cycles of correlating expression data with model to improve model and OPH.

Relevance to Nerve Agents?
Sent to Steve Harvey:
MCS (i.e., cell-free extract control), OPH, T26M, T171I-His, C199M-His, C199M-T171I-His and C199M-T26M-His.

Synergy of coarse-grained modeling + Quantum Mechanical Modeling of Active Site?