Discovery of External Modulators of the Fe-Fe Hydrogenase Enzyme in *Clostridium acetobutylicum*

by Michael S Lee, Margaret M Hurley, Matthew Servinsky, and Christian Sund

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Discovery of External Modulators of the Fe-Fe Hydrogenase Enzyme in \textit{Clostridium acetobutylicum}

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Complex metabolic networks contain many essential enzymes that cannot be genetically altered or deleted without adverse impact on the host cell. We have developed an alternate strategy to modulate metabolite flow through these essential enzymes with the goal of more efficient production of commodity chemicals (i.e., biofuels), better waste remediation, and novel antibiotics. In this report we tackle the first of 2 goals to computationally discover and design small molecules and modified bacterial genes (for producing peptide tails to a common bacterial protein) that will inhibit native bacterial metabolic enzymes and alter metabolic output. We computationally screened the million-compound ChemDiv catalogue and identified and purchased 20 candidate inhibitors to 2 sites of the Fe-Fe hydrogenase protein in Clostridium acetobutylicum. During this effort, we developed a novel assay for detecting gas production in ultrasmall bacterial cultures and verified this assay with TNT as a positive control. After testing all of the candidate molecules in this assay, we found some that moderately reduced hydrogen gas production as desired and some that reduced overall cell viability.
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1. Introduction

Complex metabolic networks contain many essential enzymes that cannot be genetically altered or deleted without adverse impact on the host cell. We envision an alternate strategy to modulate metabolite flow through these essential enzymes, which will enable unprecedented metabolic control, leading to more efficient production of commodity chemicals (i.e., biofuels), better waste remediation, and novel antibiotics. Our 2 goals are to computationally discover and design small molecules and modified bacterial genes (for producing peptide tails to a common bacterial protein) that will inhibit native bacterial metabolic enzymes and alter metabolic output. In this one-year effort, we attempted to inhibit, via small molecules, the essential hydrogenase (HydA) (Fig. 1) of *Clostridium acetobutylicum* (*C. acet.*),\(^1\)\(^2\) which is predicted to subsequently increase butanol solvent production by the organism.\(^3\) Unmodulated wild-type *C. acet.* yields a 3:6:1 ratio of acetone, butanol, and ethanol. External modulators in the form of small molecules would provide a proof of principle that we can inhibit the HydA and shift metabolism toward butanol production.

![Fig. 1 Hypothetical internal peptidic inhibition of bacterial HydA](image)

This report provides a structural template for a future study: the design of small peptides to inhibit in the active site of the HydA. This peptide sequence, in turn, may be inserted at the head or tail of the sequence of an abundant bacterial protein to result in an engineered inhibitor of the HydA. The benefit of this approach is that the concentration of the inhibitory peptide will be directly tied to the presence of a commonly expressed bacterial protein (CEBP). As a bacterium undergoes stress from reduced HydA activity, expression of the CEBP will drop, which in turn will reduce concentration of the designed inhibitory peptide, thus creating a feedback loop that will limit cell stress and death.
2. Objectives

The objectives in this report are the following:

- Perform a computational screen of one million compounds against 2 distinct surface regions of the C. acet. HydA protein structure (based on Peters et al.4) and select a total of 20 compounds for experimental testing.
- Using experiment, determine if any of the 20 small molecules inhibit the bacterial HydA, thereby reducing hydrogen output.

The following are future objectives:

- Analyze chemical functionality and predicted binding mode of all of the small molecule hits to design small peptides that will inhibit the active site.
- Genetically engineer the top peptide candidates into the C-terminus (or N-terminus) of an abundant cytoplasmic protein.
- Test the bacterial system for viability and hydrogen production.

Successful completion of these goals would be a strong scientific result, yielding future research directions for the US Army Research Laboratory (ARL) biotechnology program and strengthening the repertoire of ARL’s Computational and Information Sciences Directorate and Sensors and Electron Devices Directorate (SEDD) scientific tools for collaboration with life and biomaterials scientists within the Department of Defense and external institutions.

3. Methods

3.1 Computational Modeling of the Protein and Ligand

We used various modeling tools to determine the correct site for screening inhibitors of the enzyme. The methods we used are described in detail in Section 4 of this report.

3.2 Computational Docking of Small Molecule Inhibitors

The primary challenge in this report is the computational discovery of small molecule inhibitors. There are tens of millions of commercially available small compounds (molecular weight: <500 Daltons) that can be potentially experimentally tested to reduce HydA activity in bacteria. Computational
Prescreening not only greatly improves the cost/benefit analysis by an order of at least 1,000, but it also reduces the likelihood of false positives from assay signal detection error and unintended binding sites.

We used an automated computational pipeline developed in-house that employs Autodock Vina\textsuperscript{5} to dock small molecules to a homologous structure of the \textit{C. acet.} HydA from \textit{Clostridium pasteurianum} (\textit{C. past.}; protein data bank [PDB] id: 1FEH\textsuperscript{1}) (Fig. 2). Our group has successfully used similar screening protocols to find small molecule inhibitors of host/virus and virus/virus interactions in Ebola and Marburg viruses\textsuperscript{6–9} and an ATP (adenosine triphosphate)–powered secretion system in plague bacteria.\textsuperscript{10} Two million commercially available compounds from the ZINC-processed ChemDiv catalog\textsuperscript{11} were screened in 2 separate sites on the protein surface. The docked molecules were scored by consensus of the Vina scoring function and the third-party DrugScore DSX function.\textsuperscript{12} The top 100 molecules from each of the 2 docking sites were visualized and 10 molecules from each screen were selected for purchase. See the Appendix for the structures of the ZINC molecules tested.

\begin{figure}
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\includegraphics[width=\textwidth]{Fig2.png}
\caption{Atomic structure of closely related \textit{C. past.} iron (Fe)-HydA protein\textsuperscript{4}}
\end{figure}

\subsection*{3.3 Computational Docking of Peptide Inhibitors}

Docking of biological moieties such as peptide-protein complexes has long been recognized as presenting a challenge because of a variety of issues including extreme flexibility of both components,\textsuperscript{13} the transferability of the scoring function (the expression used to assess the energy of the system), and the difficult necessity of a blind approach to determining a complex where little is known beyond the structure of the protein and the sequence of the peptide inhibitor. Our group has successfully explored these issues in recent work developing peptide
binders to Anthrax Protective Antigen and Anthrax Lethal Factor,\textsuperscript{14} in which it was demonstrated that a combination of molecular dynamics and standard docking protocols helps mitigate these challenges.\textsuperscript{15,16}

Inhibition of the \textit{C. acet.} Fe-HydA has largely been studied from the perspective of small molecule inhibitors such as trinitrotoluene (TNT). Development of peptide-based inhibitors requires combining information from the small molecule inhibitors developed as outlined previously with information obtained from interactions of HydA with proteins such as ferredoxin (Fd), the physiological electron donor of Fe-HydA in \textit{C. acet.}\textsuperscript{17} In this case, an additional level of complexity is introduced because of the absence of an experimentally derived structure of either \textit{C. acet.} HydA or Fd. Both proteins are sufficiently similar to their counterparts in \textit{C. past.} to allow development of suitable homology models. A model of the docked HydA:Fd complex was then developed and analyzed. A suitable peptide moiety was pared down from the Fd component to provide a reasonable starting guess at a peptide inhibitor for the HydA. The peptide was built, simulated with molecular dynamics, docked to HydA, and analyzed in the context of the successful small molecule inhibitors determined in the first part of this project.

### 3.4 Development and Testing of Gas Chromatography Assay

A new protocol has been established to test the effects that potential HydA inhibitors may have on gas production in \textit{C. acet.} fermentations. Typically, gas production is monitored at 15-min intervals throughout the fermentation using gas chromatography. The minimum volume for running our bioreactors is normally 0.5 l. Thus it is not practical, given limited availability (<10 mg) of several of the compounds, to screen using this system. To circumvent this obstacle, we developed a protocol to perform these experiments in sealed serum vials and take endpoint measurements of the headspace with a micro gas chromatograph. For this protocol, fermentations were started in an anaerobic chamber containing an atmosphere of 100\% nitrogen gas (N\textsubscript{2}). An additional anaerobic chamber was set up specifically for these experiments, as the atmosphere of the chamber used for routine growth contains N\textsubscript{2}, carbon dioxide (CO\textsubscript{2}), and hydrogen gas (H\textsubscript{2}). The concentrations of CO\textsubscript{2} and H\textsubscript{2} were measured after the fermentation was complete. By comparing H\textsubscript{2}:CO\textsubscript{2} ratios with control fermentations it can be determined if a test compound inhibits the HydA.

To verify the method, we tested TNT since it has been demonstrated to inhibit hydrogen production from \textit{C. acet.} fermentations in our laboratory. Preliminary experiments indicated that we were able to measure a 30\% reduction in hydrogen
production from fermentations containing 500-µM TNT compared with the negative control. TNT was used as a positive control in all HydA inhibitor screens to ensure that our system was continuing to perform correctly.

4. Results and Discussion

4.1 Structural Model of the Hydrogenase

We tried to generate an accurate model of HydA by performing a careful analysis on a wide array of homology models (Fig. 3). Multiple comparative modeling algorithms were tested for the construction of a *C. acet.* HydA homology model, including SWISS-MODEL, CPHmodels, and I-TASSER. Agreement among these models was excellent, as well as agreement with the *C. past.* crystal structure (PDB id: 1FEH). Alignment and comparison with the 1FEH structure gave a backbone root mean squared deviation (RMSD) of 3.44 Å for the CPHmodels algorithm, 3.30 Å for I-TASSER, and 3.32 Å for SWISS-MODEL. In addition to an excellent RMSD, I-TASSER was the only one of these algorithms that did not truncate the *C. acet.* HydA structure to agree with the somewhat shorter *C. past.* protein. I-TASSER was also used to develop a homology model for the *C. acet.* Fd based upon the *C. past.* crystal structure (PDB ID: 1CLF).

Unfortunately, we found that protein-protein docking using comparative models of this resolution can lead to anomalous results whereby side chains are not in the right place or, worse, entangled with each other. For the computational screening of compounds, we chose to use the *C. past.* structures of Fd and HydA as is. The
risk was that we could have found the right molecules for the wrong protein. However, we observed good sequence similarity between *C. past.* and *C. acet.* HydA in the docking target regions we have chosen.

### 4.2 Protein-Protein Docking to Determine Sites for Inhibitors

To determine the sites to computationally dock to, we used 2 well-regarded protein-protein docking programs, ZDOCK\(^1\) and PatchDock,\(^2\) to model how the 2 proteins connected to each other\(^17,23,24\) (Fig. 4). With a significant amount of molecular surface area in the HydA, we found at least 3 putative docking locations for the much smaller Fd protein. Of these 3 locations, 2 provided a close approach between the 2 Fe-sulfur (S) clusters of Fd (blue) and the Fe-S cluster “wire” in the HydA (red). Close approach is required for electron transfer from the Fd “shuttle” to the HydA “port”. The electrons absorbed by the HydA wire are used to drive the chemical reaction, \(2H^+ + \text{Fd (reduced)} \rightarrow 2H_2 + \text{Fd (oxidized)}\), which results in more \(H_2\) output and less overall butanol production in the bacteria.

![Fig. 4](image)

**Fig. 4** Putative docking site 1 of Fd (blue) to Fe-only HydA (red); Fe-S clusters are green and yellow

### 4.3 Computational Small Molecule Docking of the 2 Sites

For each of the 2 possible docking sites, we computed a midpoint coordinate for a cubic box with a side length of 20 Å. We then extracted the database of 3-dimensional structures of compounds from the commercial vendor, ChemDiv
(San Diego, CA), from the ZINC database. With roughly 1.2 million entries, we filtered out molecules with predicted log partition coefficients $>4.0$ to ensure that we did not consider “greasy” molecules that tend to stick to many proteins and could cause unintended inhibition of other bacterial processes. The remaining approximately 1 million molecules were computationally docked with Autodock Vina within the specified boxes. The molecules were free to flex around their torsional angles, and often several docking structures were generated for each compound. Twenty structures for each of the approximately 1 million candidate molecule were scored with the third-party DrugScore program. The scores from Vina and Drugscore were normalized and summed to yield a Z-score that was used to rank the best molecules. We visualized the top 50 scoring molecules for each docking site, and chose the structures that looked best by expert opinion. The top 10 compounds from each site were labeled by their original ChemDiv catalog number and the lists were sent to the SEDD experimental team.

### 4.4 Computational Peptide Docking of the Entire Protein

Analysis of the Patchdock-generated HydA:Fd complex showed a tightly folded single loop of the Fd consisting of residues 21–33 (sequence: NAISQGDSIFVID) to be situated within the site 1 crease of the HydA in close proximity to the Fe$_2$S$_2$ cluster. This was taken as an excellent starting point, and a peptide of that sequence was built, solvated, minimized, and simulated at normal pressure and temperature of 1 atm and 300 K for 40 ns with a time step of 2 fs using the NAMD software. It was apparent during the course of the simulation that this single-fold configuration of the peptide is moderately stable, as the strand unfolded and refolded multiple times. Multiple configurations reflecting both folded and unfolded states were selected from the simulation trajectory and docked to HydA using Patchdock. This represents a global dock on the entire HydA structure.

Ranked analysis of the results showed a “hotspot” of folded configurations within the site 1 crease, as expected, while an additional hotspot for unfolded configurations was seen along the side leading to site 2 (Fig. 5). Electrostatic analysis with the APBS software of Baker et al. highlights the co-localization of electropositive (blue) residues in each of these sites, allowing favorable interaction with peptide electronegative residues at the end and center. The folded configuration of the peptide binding at site 1 allows the segregation of a cluster of hydrophobic residues at one end of the peptide onto the protein interface while allowing solvent exposure of hydrophilic residues clustered on the upper fold of the peptide. The unfolded peptide configurations fit more neatly into the extended patch of electropositive surface residues adjacent to site 2. There are interesting
structural and chemical similarities between the folded peptide configuration and small molecule inhibitors determined in the first part of the project. Both sets of inhibitors are approximately the same size and shape for fitting the site 1 patch shown in Fig. 5, occupying a slightly bent but overall rectangular volume of roughly 20 Å on the long axis. The chemical character of, for example, small molecule candidate inhibitor ZINC21938598 is analogous to the lower fold of the peptide, consisting of electronegative groups at each end suitable for binding to the protein with a string of hydrophobic aromatic rings in the middle. We propose that the folded configuration of the peptide is optimum for binding, as it aggregates hydrophobic residues on one side and hydrophilic on the other. The logical next extensions of this work are to explore mutations to further stabilize this folded configuration and to make single-point mutations on the binding side of the fold to further enhance binding.

Fig. 5 Peptide docking on HydA shows hotspots at site 1 (central right) and side entrance to site 2 (lower left). Electrostatic surface of protein was generated with APBS software.27
4.5 Testing for Hydrogenase Inhibition

In the first round (Fig. 6), we tested all 20 compounds as previously described at 500 µM and found that compounds H, I, J, and T appeared to reduce hydrogen production in these fermentations. We also noted that compounds F and N killed the bacteria, which led us to perform a second round of testing at lower concentrations (Fig. 7) to ensure the reason for lethality was not due to too strong of a reduction of HydA activity.

Fig. 6  H₂/CO₂ ratio after incubation with small molecules
Fig. 7 Representative results from tests for HydA inhibition. (top) Gas concentrations in culture headspace after 6 h of incubation with test compounds. DMSO was used a solvent for the test compounds and serves as a negative control. TNT is a positive control for the assay. (bottom) Gas concentrations form cultures receiving dilutions of compound F.

After a second round of testing (Fig. 7; data not shown), we concluded that none of the molecules selected by computational screening provided a statistically significant reduction in H$_2$ production. In the future, we will look at a few of the following possibilities:

- Crystallization and X-ray structure determination of the actual $C. acet.$ Fe-HydA protein. This protein is of sufficient interest to the hydrogen fuel production community to be worthwhile to characterize more carefully.

- Experiments to determine the exact binding site of Fd to the HydA. Not only would this provide a site for computational docking, but it would also help researchers understand how electrons flow from the Fd to the HydA.

- Computational screening of the new protein structure or computational screening of a sufficiently accurate homology model.

- Selection and purchase of peptide fragments resembling the Fd protein as potential peptide inhibitors of the HydA.
5. Conclusions

A new experimental assay for determining H$_2$ production in ultra-small bacterial cultures was developed and verified with TNT. In addition, small molecule inhibitors of *C. acet.* Fe-HydA were computationally selected. Moreover, a peptide inhibitor has been designed computationally from the Fd:HydA complex that shares attributes of the selected small molecules. Several of the putative small molecule inhibitors were found to reduce H$_2$ production moderately, but not significantly. Other molecules actually reduced cell viability. Future directions were suggested.
6. References


7. Transitions

At this time, no publication or presentations of this work beyond this report are planned. However, we fully expect scientific results and technological improvements from this work will inform future endeavors in our respective laboratories.
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Appendix. Small Molecules Experimentally Tested

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