Rational Design and Characterization of a Bacterial Redox Enzyme

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Introduction

Steroids

- Recalcitrant to degradation because they are hydrophobic and lack functional groups[1]
- Adverse effects on body and environment[1]
- Important for proper development, fertility, drugs, and cell signaling.
- Despite their lack of reactivity, many microbes have evolved catabolic pathways for the complete degradation of steroids.

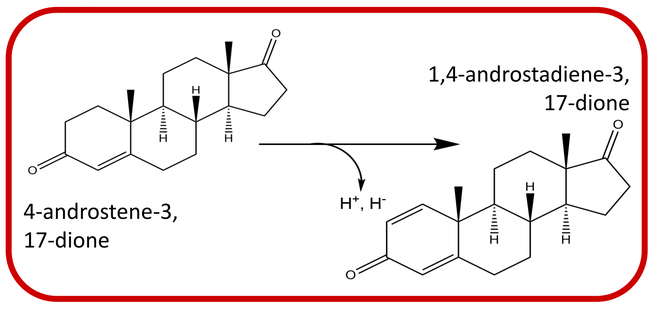


Fig. 1 Native reaction catalyzed by enzyme.

- This study presents the rational design and characterization of a flavin adenine dinucleotide (FAD)-dependent bacterial oxidoreductase that is important for steroid degradation in microbes.
- This enzyme was identified for its promise in the development of a biosensor for cortisol, although it has a higher activity against progesterone rather than cortisol.[2]

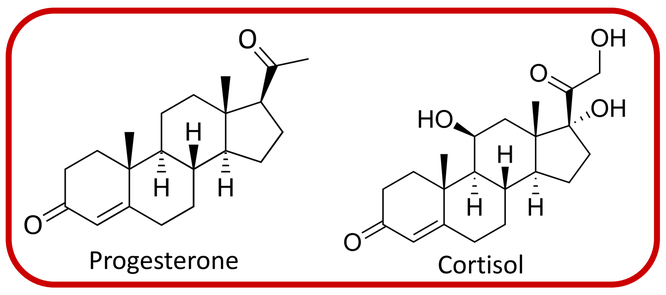


Fig. 2 Structures of steroid substrates accepted by the enzyme.

- This goal of this study is to shift the bacterial enzyme's substrate specificity from progesterone to cortisol, and to understand how the mutations affect substrate specificity.

Methods

Rational Design of Variants

- Proposed rationally designed variants using AlphaFold-predicted structures.
- Performed PCR site-directed mutagenesis (SDM) on the gene for the enzyme to incorporate amino-acid side chains that would form new hydrogen bonds with unique hydroxyl groups found on cortisol that are not present in progesterone.

Expression and Purification

- Molecular cloning, Mini Prep, Test Expression
- Large scale growth and heterologous expression for purification and characterization

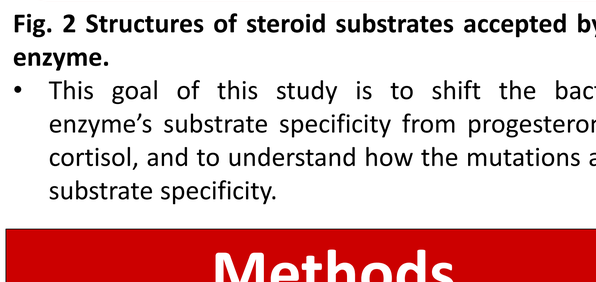


Fig. 3 SDM Process. Created with BioRender.com

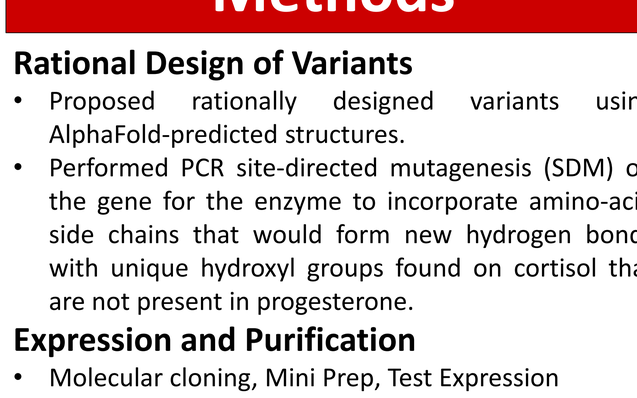


Fig. 4 Purification process. Created with BioRender.com

Protein Purification: SDS-PAGE

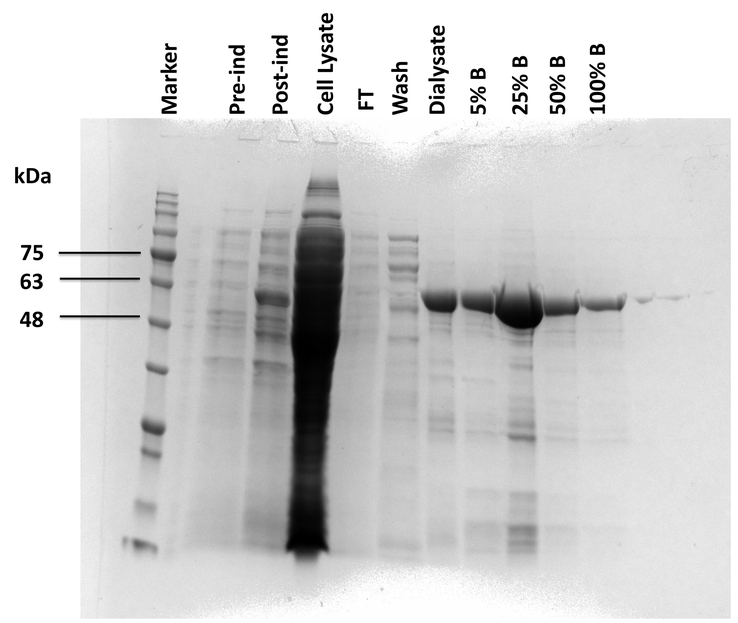
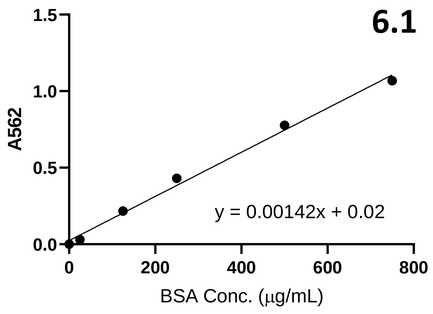


Fig. 5 Variant 1 was successfully purified as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pre- and post-induction lanes show the solution during cell growth. The cell lysate was obtained after microfluidizing the cells. The following lanes were obtained during nickel affinity chromatography. Since the enzyme is around 54 kDa, the band below 63 kDa was identified as Variant 1.

Protein Quantitation



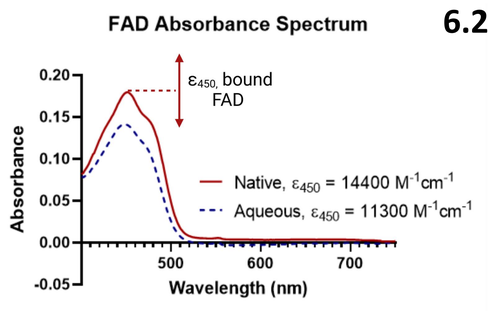
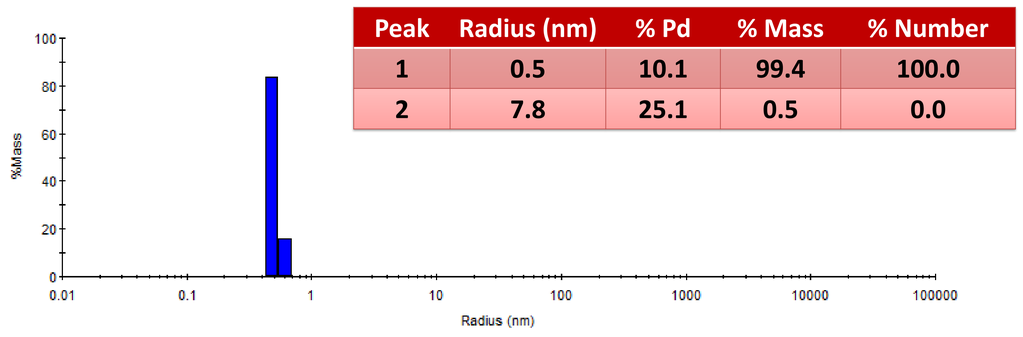


Fig. 6.1 and Fig. 6.2 The total protein concentration in solution was determined using two spectrophotometric assays: the bicinchoninic acid (BCA) assay with a bovine serum albumin (BSA) standard curve. Extinction coefficients for FAD for bound FAD were determined to find the active concentration. The total protein concentration for Variant 2 was 297 µM, and the active protein concentration was 226 µM, making the FAD occupancy 76%.

Dynamic Light Scattering



Peak	Radius (nm)	% Pd	% Mass	% Number
1	0.5	10.1	99.4	100.0
2	7.8	25.1	0.5	0.0

Fig. 7 The percent polydispersity for Variant 2 is 10.1% with a percent mass of 99.4%. By scanning a small sample of the protein, more characteristics of it are known. The large peak and narrow distribution signify that the solution is very monodisperse (i.e., most of the molecules are very similar in size). This shows that most of the protein is folded properly.

Steady-state Kinetics

Fig. 8.1 DCPIP Assay. By mixing the enzyme, substrate, and 2,6-Dichlorophenolindophenol (DCPIP), the decreasing absorbance of DCPIP over time is recorded. In turn, this shows the concentration of DCPIP over time, which can be converted to concentration of product over time. This describes the velocity of the reaction between DCPIP and the enzyme, which can be graphed against substrate concentration. This is how the k_{cat} and K_m values are determined for each substrate. **Fig. 8.2 Variant 1 showed no significant results regarding specificity towards progesterone and cortisol.**

X-Ray Crystallography

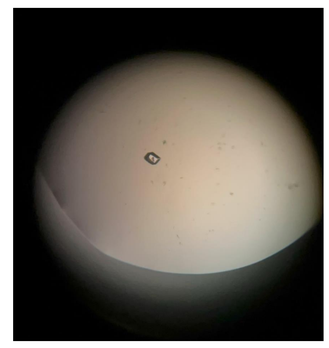


Fig. 9 A clear, cubic crystal.

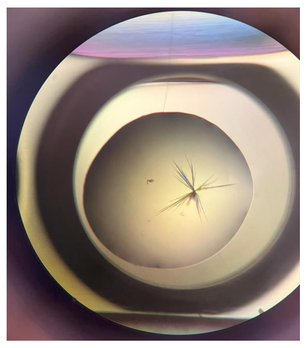


Fig. 10 A needle cluster.

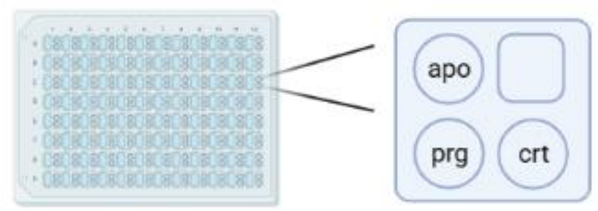


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Fig. 11 Two crystal trays were set up, one with PEG Ion conditions and one with HT Index.

Results / Discussion

Table 1. Kinetics results describing substrate specificity.

Variant	k_{cat}/K_m ($M^{-1}s^{-1}$)		prg/crt (k_{cat}/K_m)
	Progesterone	Cortisol	
WT	$2.5x10^5$	$3.2x10^4$	7.8
Variant 1	$2.83x10^5$	$2.3x10^4$	12
Variant 2	$2.0x10^4$	$6.0x10^1$	330

- **Variant 1** – Since this variant did not show more than one magnitude of change from WT in the k_{cat}/K_m values for both substrates, the results were deemed insignificant and not different from WT.
- **Variant 2** – This variant did show significant change in the k_{cat}/K_m values for both progesterone and cortisol, as the k_{cat}/K_m for cortisol decreased by 3 orders of magnitude, and the value for progesterone decreased by one order of magnitude. Although k_{cat}/K_m has decreased compared to WT, it is still three orders of magnitude higher than that of cortisol, so the enzyme is still very specific to progesterone.
- **Crystal conditions** – The HT Index high-throughput screen produced the best crystals.

Conclusion / Future Work

Further work is needed to develop a biosensor for cortisol. By using variants that are more specific to progesterone in combination with variants that have activity against cortisol, a multiplex sensor for cortisol can be developed.

Conditions from the HT Index screen can be optimized. This will help produce more ordered crystals, which could lead to determining the structure of the enzyme.

References

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2– Grazon, C., Baer, R.C., Kuzmanović, U. *et al*. A progesterone biosensor derived from microbial screening. *Nat Commun* 11, 1276 (2020). https://doi.org/10.1038/s41467-020-14942-5

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