Studies of Cysteine 298 Role in [FeFe]-hydrogenases

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INTRODUCTION

Cysteine 298 in *Clostridium acetobutylicum* HydA hydrogenase (CaHydA) is a highly conserved residue in all [FeFe]-hydrogenases and it is one of the residues that are closer to the Fe_d atom of the H-cluster. It has previously been proposed to be involved in the catalytic cycle during proton transfer and to be an important structural element of the cavity that hosts the active site H-cluster.

In order to obtain a comprehensive study of its role, a semi-random approach was used. C298 was studied by site saturation mutagenesis, to simultaneously evaluate the functional effect of cysteine replacement with all the other 19 aminoacids. A specific activity screening protocol was developed in order to test the effect of the mutations directly in libraries composed of a large number of *E. coli* clones and the most relevant variants have been characterised.

CYSTEINE 298 IN [FeFe]-HYDROGENASES

Cysteine 298 is part of the L1 motif (TSCCPxW) and it is highly conserved in all [FeFe]-hydrogenases. It is located very close to the Fe_d atom of the H-cluster. For these reasons it might have several roles in structure/function, catalytic mechanism and oxygen sensitivity.

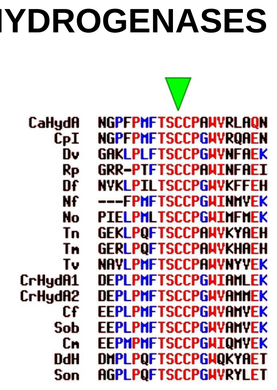

Figure 1. Sequence alignment of various [FeFe]-hydrogenases.

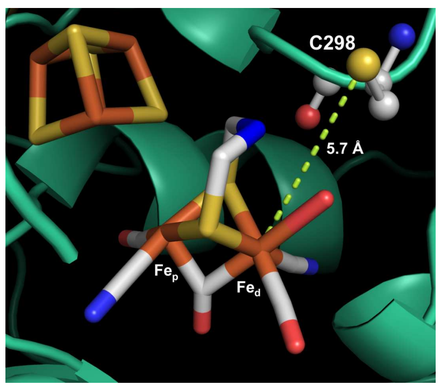

Figure 2. Structure model of CaHydA with the H-cluster and cysteine 298.

ACTIVITY SCREENING PROTOCOL

A specific screening protocol was set up to directly assay [FeFe]-hydrogenase activity in *E. coli* colonies. The screening takes advantage of methyl viologen (MV) reduction coupled to H_2 uptake by the enzyme. The specificity and the lowest detection threshold were also tested.

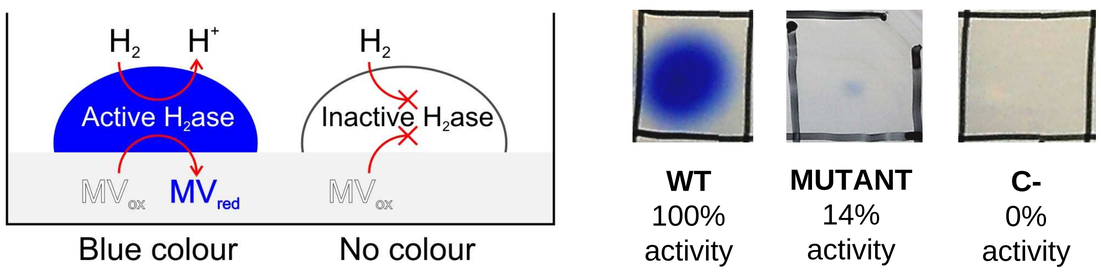

Figure 3. Scheme of the activity screening mechanism and representative tests demonstrating the specificity and the lowest detection threshold.

RANDOM LIBRARY GENERATION AND SCREENING

The site saturation library was generated using an NNK degenerated codon, thus allowing the generation of 32 possible DNA variants, encoding for all the 20 aminoacids and requiring a reasonably low screening effort. Screening of 315 clones allowed to cover all the possible variants with a probability of completeness of 99.79%.

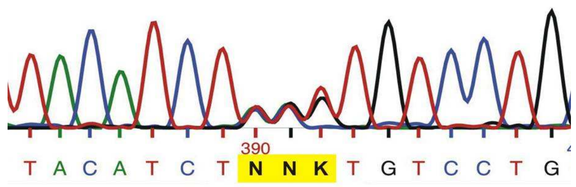

Figure 4. DNA sequencing of the plasmid library showing the expected randomisation.

The screening revealed that most CaHydA variants did not show any detectable hydrogenase activity, while some showed *wild type*-like signals. No clones with an intermediate signal could be observed. DNA sequencing revealed that the active clones were variants encoding for cysteine (codon TGT) or aspartic acid (codon GAT) only.

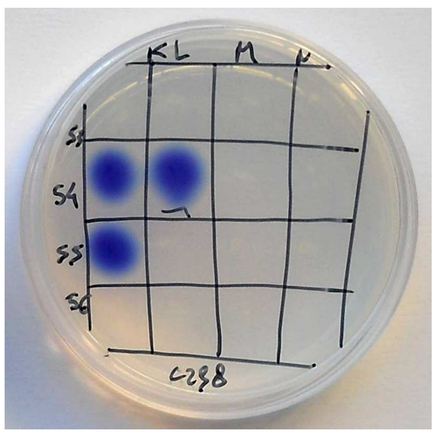

Figure 5. Representative set of the screened clones including CaHydA WT as a control (L54).

Sequence	Codon	Amino acid	Clones	Frequency	Screen
	GAT	Aspartic acid	8	2.5%	Active
	TGT	Cysteine	9	2.9%	Active
	AGT	Serine	-	-	Negative
	TCG	Serine	-	-	Negative
	TCT	Serine	-	-	Negative
	AAT	Asparagine	-	-	Negative
	ACT	Threonine	-	-	Negative
	ACG	Threonine	-	-	Negative
	CAG	Glutamine	-	-	Negative

Sequence	Codon	Amino acid	Clones	Frequency	Screen
	CAT	Histidine	-	-	Negative
	AAG	Lysine	-	-	Negative
	CGT	Arginine	-	-	Negative
	ATG	Methionine	-	-	Negative
	TTT	Phenylalanine	-	-	Negative
	GCT	Alanine	-	-	Negative
	ATT	Isoleucine	-	-	Negative
	GGT	Glycine	-	-	Negative
	GGG	Glycine	-	-	Negative

Figure 6. DNA sequencing of all the active clones and of a representative subset of negative clones, demonstrating the diversity of screened clones.

PURIFICATION AND CHARACTERISATION OF SELECTED VARIANTS

Five variants were selected on the basis of the screening results and structural similarity and compared to the *wild type* enzyme. Recombinant expression in *E. coli* and affinity purification were accomplished under strict anaerobic conditions taking special care to decrease variability.

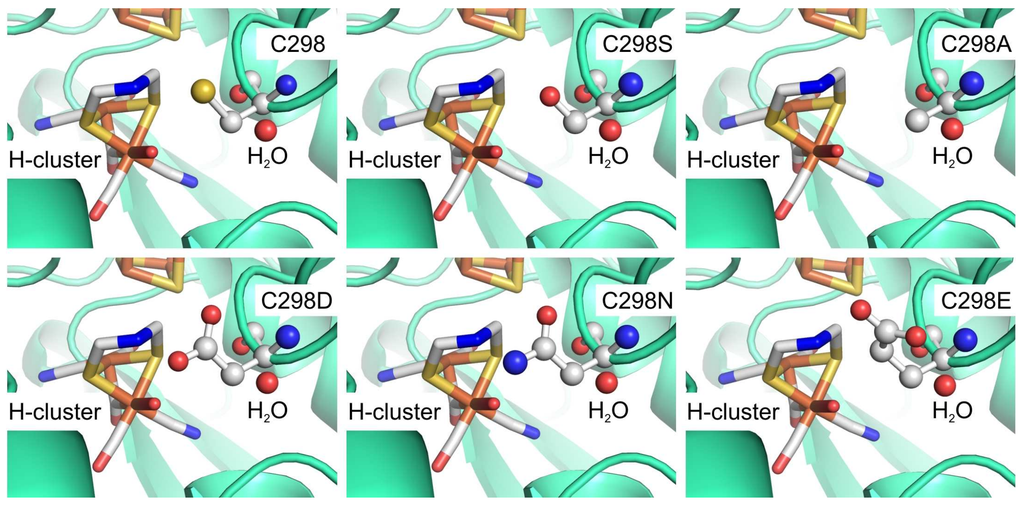

Figure 7. Summary of the 5 variants selected and the *wild type* enzyme. Models of the structures were obtained using PyMol.

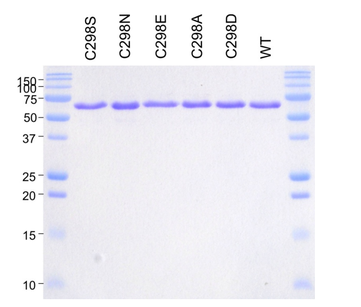

Figure 8. Coomassie stained SDS-PAGE of CaHydA WT and the 5 selected variants (65.5 kDa).

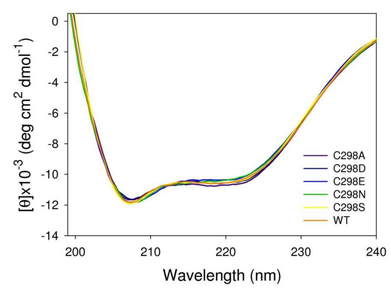

Figure 9. Far UV circular dichroism spectra, showing no major structural effect due to the mutation in the selected variants.

The secondary structure was not importantly affected, as determined by circular dichroism spectroscopy in the far UV region.

The enzymatic activity assays confirmed the results obtained during the library screening: only C298D had a remarkable activity (50% in comparison to the WT). C298E and C298S showed very little residual activity, while C298N and C298A did not show any detectable activity. Also, C298D caused a shift in the pH activity profile towards acidic conditions.

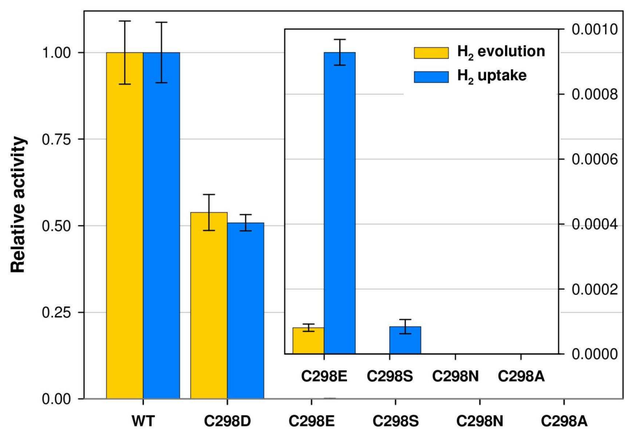

Figure 10. Relative hydrogenase activity with methyl viologen as artificial electron mediator. H_2 evolution was assayed by gas chromatography; H_2 uptake was assayed spectrophotometrically. Relative activity was calculated as the ratio with the WT activity.

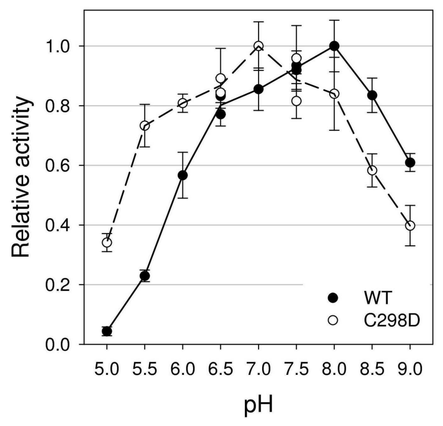

Figure 11. Hydrogen evolution activity dependence of the pH.

CONCLUSIONS AND FUTURE PERSPECTIVES

The exclusive functional replacement of cysteine with aspartic acid, an ionisable residue, and the shift in the pH activity profile demonstrate experimentally the central role of C298 in the proton transfer pathway to the active site during [FeFe]-hydrogenase catalysis. Since C298 is strongly conserved among all the known functional [FeFe]-hydrogenases, it is reasonable that the results obtained here for CaHydA have broader implications regarding its functional significance in the entire class.

Future work will aim at a more detailed characterisation of the most relevant variants, application of site saturation mutagenesis to other residues within the enzyme core and study of other [FeFe]-hydrogenases.

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