Stabilization of cyclohexanone monooxygenase by a computationally designed disulfide bond spanning only one residue

Beek, Hugo L. van; Wijma, Hein J.; Fromont, Lucie; Janssen, Dick B.; Fraaije, Marco W.

Published in:
FEBS Open Bio

DOI:
10.1016/j.fob.2014.01.009

Publication date:
2014

Supplementary data

Table S1 List of *in vitro* tested mutants, with the apparent melting temperatures determined in initial screening (from 2.5 mL scale purification and including 20 mM imidazole). n.e.: not expressed. The listed values differ from the final values (see text main manuscript) as enzyme concentrations in this experiment were significantly lower, enzyme concentrations varied more between samples, and some imidazole is present.

variant	Measured T_m	Number of residues spanned by disulfide bond
wtCHMO	37.5	
A196C-T337C	37.3	140
A255C-A293C	**39.3**	**37**
A325C-L483C	**38.3**	**157**
F7C-Q34C	36.5	26
G413C-G430C	n.e.	16
I10C-A134C	35.5	123
K177C-M373C	34.5	195
K24C-S447C	34.5	422
K317C-D347C	33.5	29
K4C-K131C	36.8	126
K80C-D218C	n.e.	137
P254C-D286C	n.e.	31
P52C-A194C	n.e.	141
S2C-D130C	37	127
T415C-A463C	36.5	47
V179C-A199C	37	19
Y211C-P320C	n.e.	108
Y411C-A463C	n.e.	51
S66C-W236C	37	169
A399C-D402C	37	2
G508C-E512C	32	3
R278C-E282C	29	3
T464C-E466C	36.5	1
D311C-I314C	33.5	2
L323C-A325C	**40**	**1**
S259C-E262C	n.e.	2
V258C-E262C	32.5	3
R534stop	33.5	

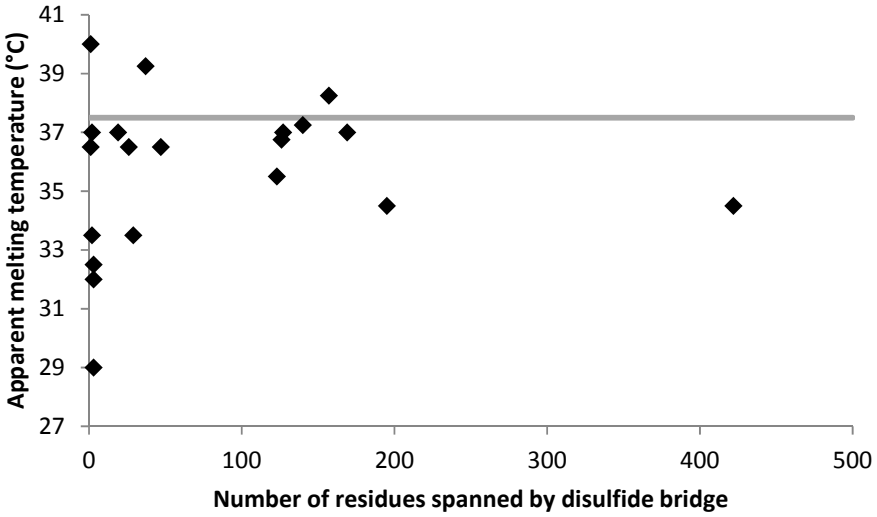

Figure S1 Apparent melting temperatures for the mutants from the initial ThermoFAD experiment (from 2.5 mL scale purification and including 20 mM imidazole) plotted against the number of residues spanned by the disulfide bridge. The grey bar represents the melting temperature of the wild-type enzyme. Values differ from the final values measured as enzyme concentrations in this experiment were significantly lower, enzyme concetrations varied more between samples, and some imidazole is present.

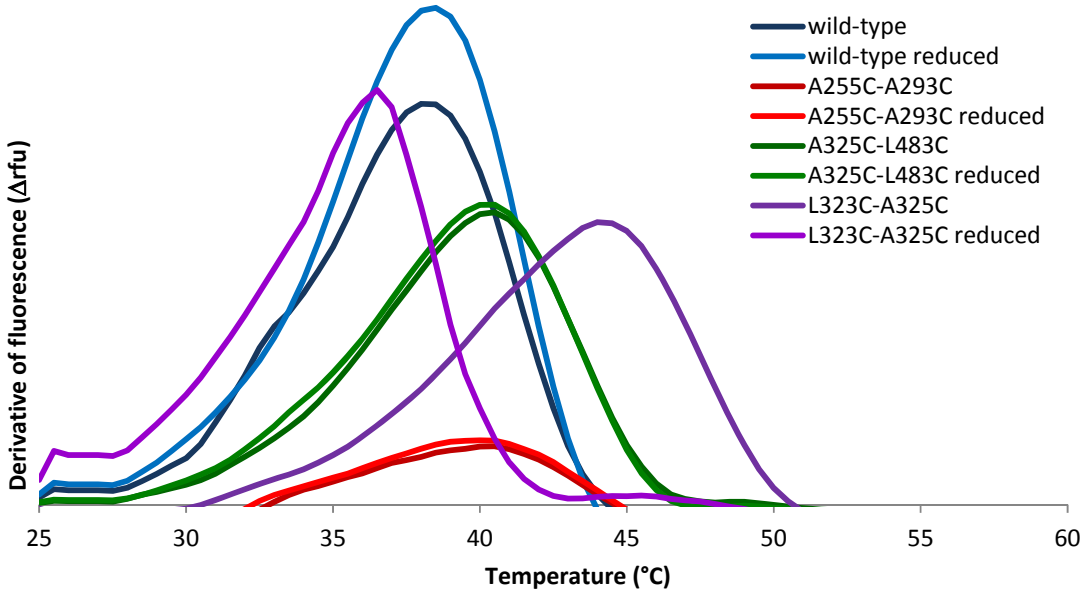

Figure S2 Graph showing the derivatives of the ThermoFAD melting curves of different enzyme variants and the variants after overnight incubation with DTT. Only the L323C-A325C mutant shows a clear shift from 44 to 36.5 °C after reduction.

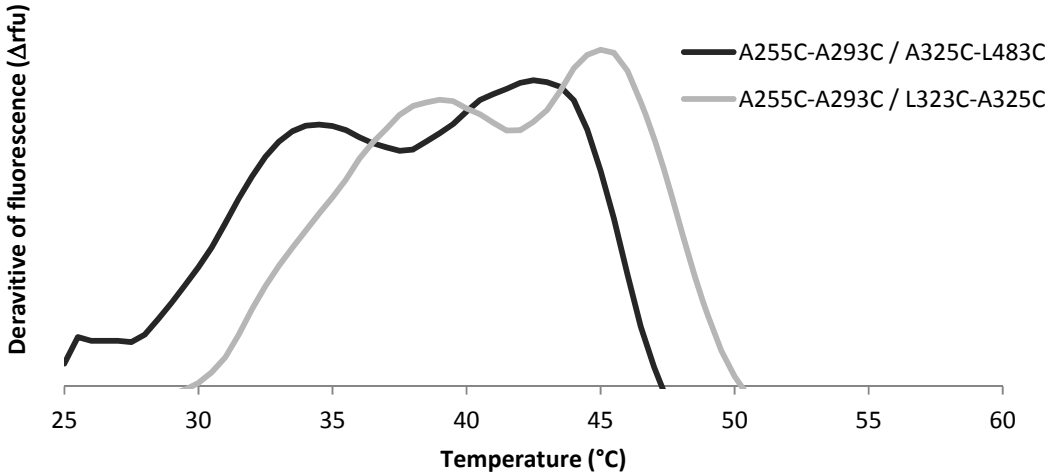

Figure S3 Graph showing the derivatives of the ThermoFAD melting curves of different enzyme variants containing 4 additional cyteines. Both variants show a slightly increased stability compared to the respective single disulfide mutants, but a significant population has a lower melting point.

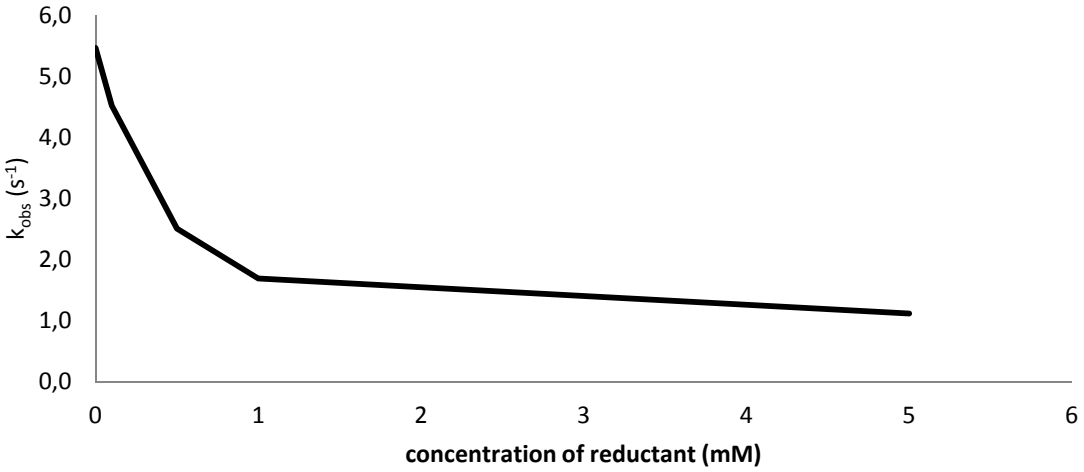

Figure S4 Inhibition of CHMO by the addition of increasing amounts DTT. Measured with 500 μM cyclohexanone and 100 μM NADPH.

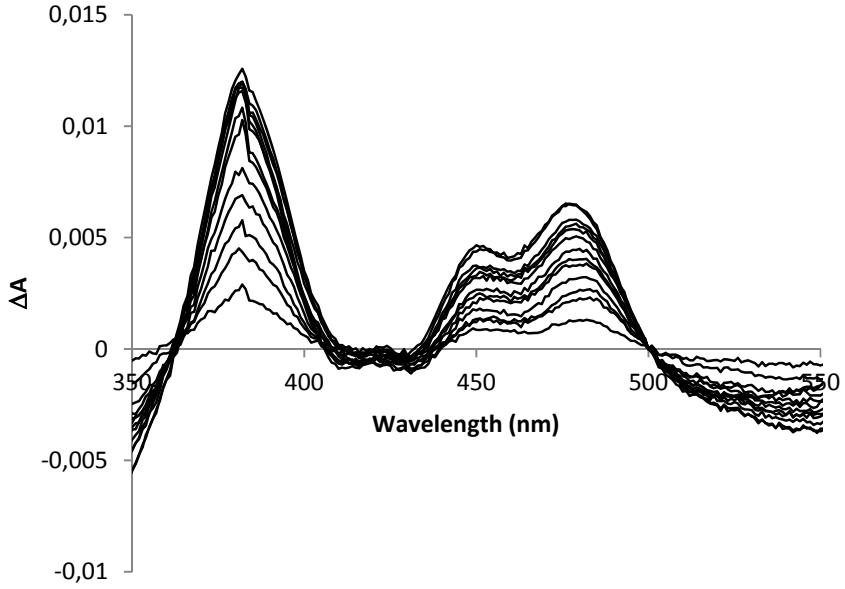

Figure S5 Graph showing the difference spectra measured every 3 minutes after addition of DTT to the L323C-A325C mutant. No significant change is observed after 24 minutes. These changes occur because the FAD spectrum is sensitive to changes in the microenvironment, which changes upon the addition of DTT.

Enzyme variant	CHMO	CHMO+DTT	L323C-A325C	L323C-A325C+DTT
K_D (μM)	28.4 ± 1.7	25.9 ± 0.1	15.4 ± 0.3	45.8 ± 13.6

Table S2 K_D values for $NADP^+$ measured by titration with $NADP^+$ for CHMO and the L323C-A325C mutant under standard and reducing conditions. Data obtained from FAD spectra corrected for dilution, scatter and absorbance of $NADP^+$. Standard deviations from two titrations.

Table S3. Steady-state kinetic parameters of the studied enzyme variants. Identical to part of Table 2, but including standard deviations and the number of experiments (between brackets). K_M values are determined from a single depletion curve and have an estimated error of < 25%.

	cyclohexanone		bicyclo[3.2.0] hept-2-en-6-one	
enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)
WT-CHMO	14.2 ± 0.008 (2)	3.6 (1)	13.4 (1)	1.3 (1)
L323C-A325C	6.1 ± 0.02 (3)	3.0 (1)	4.9 ± 0.2 (4)	0.7 (1)
WT-CHMO + DTT	13.6 ± 0.3 (2)			
L323C-A325C + DTT	9.4 ± 0.4 (3)			

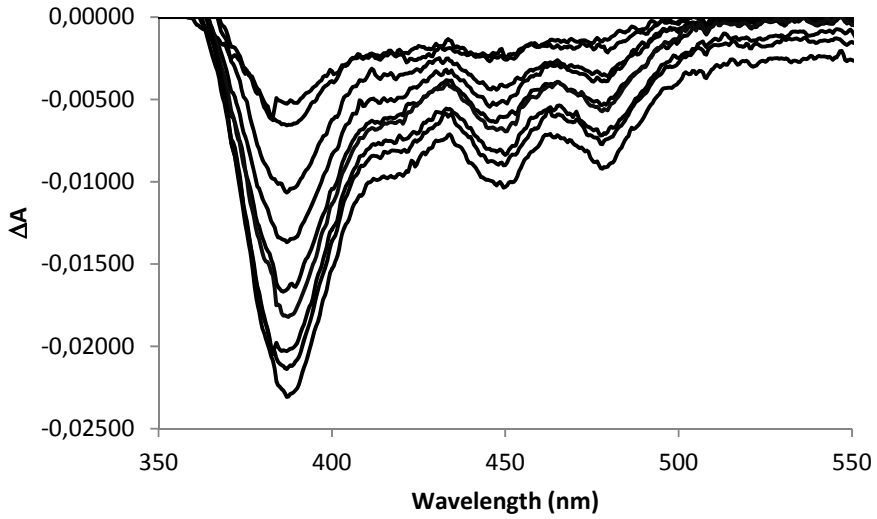

Figure S6 Typical difference spectra of enzyme with 0 to 1 mM $NADP^+$. 7.2 μM enzyme was used, corresponding to 0.1 A at 440 nm.

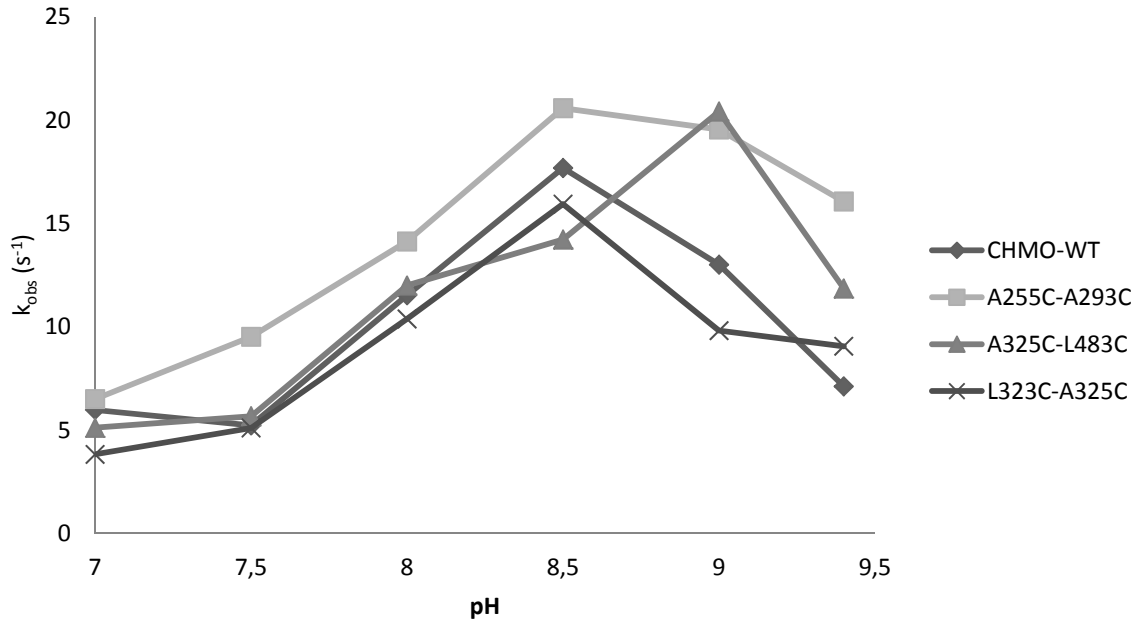

Figure S7 Graph showing the rates for CHMO and the three mutants with a higher melting point at different pH values.

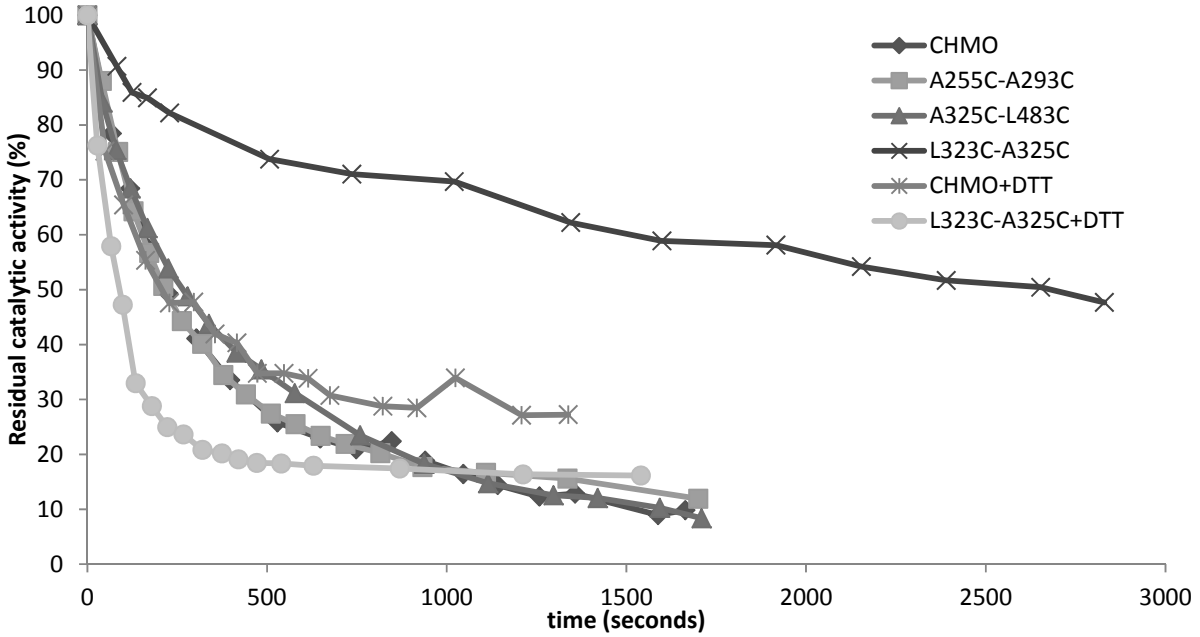

Figure S8 Enhanced stability of CHMO by an introduced disulfide bond. Enzyme was incubated at 30 °C in the presence of 0.5 mM cyclohexanone and activity was measured at different time points. The relative initial rates after addition of 100 μM NADPH are plotted with the activity at t = 0 set to 100 %. This graph includes the data already shown in figure 2.