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 concentrations 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 cysteines. 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%.

| enzyme                  | cyclohexanone |              | bicyclo[3.2.0] hept-2-en-6-one |
|-------------------------|---------------|--------------|--------------------------------|
|                         | $k_{cat}$     | $K_M$        | $k_{cat}$          | $K_M$          |
|                         | (s$^{-1}$)    | (µM)         | (s$^{-1}$)         | (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.