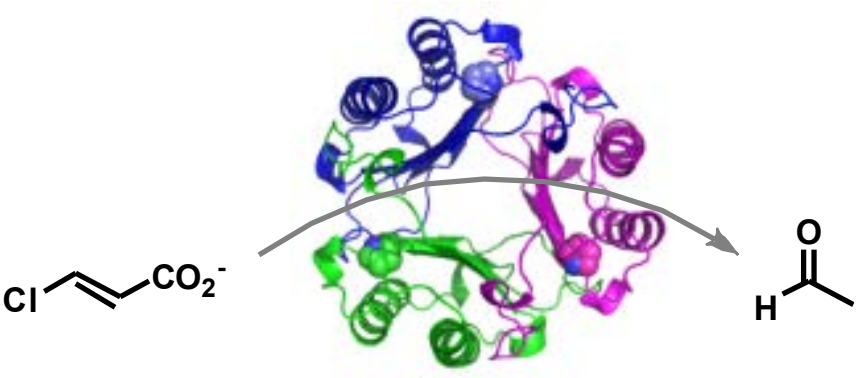
Exploring chemical versatility within the tautomerase superfamily

Baas, Jan

Chapter 3



cytokine MIF(I64V/V106L)

Dehalogenation of an Anthropogenic Compound by an Engineered Variant of the Mouse Cytokine Macrophage Migration Inhibitory Factor

Anna A.Wasiel, **Bert-Jan Baas**, Ellen Zandvoort, Wim J. Quax, and Gerrit J. Poelarends

Department of Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.

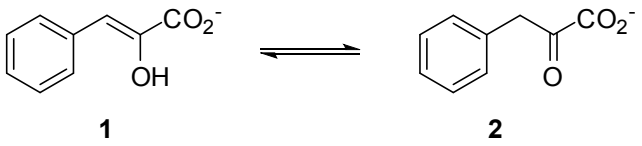
Published in *ChemBioChem* (2012) 13, 1270-1273.

Abstract

Unconventional Dehalogenase: An engineered variant (I64V/V106L) of the mouse cytokine macrophage migration inhibitory factor (MIF) promiscuously catalyzes the hydrolytic dehalogenation of the xenobiotic organohalogen *trans*-3-chloroacrylic acid to yield acetaldehyde (see picture). Although the dehalogenase activity of this MIF variant is quite low (k_{cat} = 0.36 min^{-1}), this unconventional enzyme achieves an ~10^9 fold rate enhancement (k_{cat}/k_{uncat}), matching the rate enhancements afforded by conventional enzymes that act on their natural substrates.

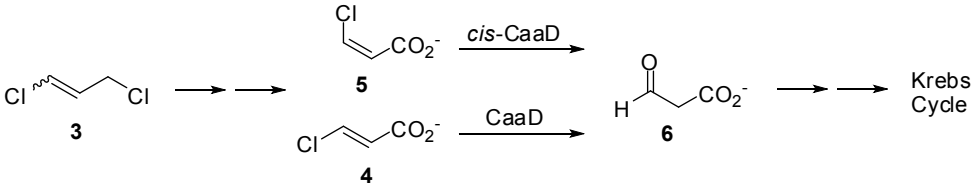
Introduction

Macrophage migration inhibitory factor (MIF) is a multifunctional mammalian cytokine that has been implicated in a number of infectious and immune responses, and is thought to play a role in conditions such as rheumatoid arthritis, sepsis, asthma, adult respiratory distress syndrome, certain types of cancer and diabetes, bowel disease, and atherosclerosis.[1] MIF is notable among cytokines because it also functions as an efficient enzyme, catalyzing the ketonization of phenylenolpyruvate (**1**) to yield phenylpyruvate (**2**) (Scheme 1).[2] This phenylpyruvate tautomerase (PPT) activity and the three-dimensional structure of MIF[3] link this cytokine to the tautomerase superfamily, a group of mainly bacterial tautomerases that is characterized by a conserved catalytic amino-terminal proline embedded within a β-α-β structural fold.[4] Indeed, the PPT activity of MIF depends on its Pro-1 residue, which has a low pK_a of ~5.6, allowing it to function as the catalytic base.[5]



Scheme 1. The tautomerization reaction catalyzed by MIF.

The enzymes *trans*-3-chloroacrylic acid dehalogenase (CaaD)[6] and *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD),[7] which are found in a bacterial pathway that degrades the synthetic nematocide 1,3-dichloropropene (**3**, Scheme 2),[8] were recently identified as new members of the tautomerase superfamily.



Scheme 2. The bacterial 1,3-dichloropropene catabolic pathway, showing the hydrolytic dehalogenation of *trans*- and *cis*-3-chloroacrylate (**4** and **5**, respectively) by *trans*- and *cis*-3-chloroacrylic acid dehalogenases (CaaD and *cis*-CaaD, respectively).

CaaD and *cis*-CaaD catalyze the hydrolytic dehalogenation of the *trans*- and *cis*-isomers of 3-chloroacrylate (**4** and **5**, respectively) to yield malonate semialdehyde (**6**) and HCl (Scheme 2).[6,7] Intriguingly, both CaaD and *cis*-CaaD exhibit promiscuous PPT activity,[9] which prompted us to initiate studies that aim to identify tautomerase members of the superfamily that possess CaaD or *cis*-CaaD activity.[10] Herein, we describe the discovery that mouse MIF has low-level CaaD activity, which can be enhanced 200-fold by two mutations (I64V/V106L) in the substrate binding pocket of this cytokine. This MIF variant achieves a remarkable rate enhancement (k_{cat}/k_{uncat} ~10^9)

as an unconventional dehalogenating enzyme. Furthermore, the finding that mammalian MIF and bacterial enzymes of the tautomerase superfamily share both dehalogenase and tautomerase activities provides evidence for divergent evolution from a common ancestral enzyme, and strongly supports the notion that the enzymatic activities of MIF are vestigial in mammals.[11]

Results and discussion

To investigate whether MIF has the ability to catalyze the dehalogenation of **4** and **5**, MIF was incubated with **4** or **5** in 100 mM phosphate buffer at pH 6.5 and 22°C, and the two reactions were monitored by ^{1}H NMR spectroscopy. Upon incubation of **4** with MIF, the intensity of the two signals corresponding to **4** decreased and four new signals appeared. Two signals (9.53 and 2.10 ppm) correspond to acetaldehyde, while the other two signals (5.11 and 1.18 ppm) correspond to its hydrate.[12] Integration of the signals indicates that ~2% of **4** has been converted to acetaldehyde and its hydrate after 7 days (Figure 1A). No product formation was detected for incubation mixtures of **5** with MIF for 7 days at pH 6.5 and 22°C. Control experiments demonstrate that the dehalogenation of **4** is an enzyme-catalyzed process. ^{1}H NMR analysis of **4** in 100 mM phosphate buffer at pH 6.5 for 14 days at 22°C showed no formation of acetaldehyde (or its hydrate), ruling out a nonenzymatic dehalogenation.

The MIF sample used in these experiments was highly purified. However, it remained possible that a trace amount of contaminating enzyme from the *E. coli* BL21(DE3) expression host could be responsible for the observed low-level dehalogenase activity. To eliminate this possibility, a mock purification was performed from BL21(DE3) cells harboring an "empty" pET20b(+) vector which lacks the MIF gene. Incubation of an aliquot of this purification with **4** for 14 days at pH 6.5 and 22°C did not result in the formation of products. Having established that MIF catalyzes the dehalogenation of **4**, kinetic parameters were determined using a previously described colorimetric assay, which monitors halide release.[13] MIF catalyzes the dehalogenation of **4** with a $k_{cat} = 3 \times 10^{-5}$ s^{-1} and a K_m of 5.8 mM, resulting in a k_{cat}/K_m of ~5×10^{-3} M^{-1} s^{-1}.

It has previously been reported that 4-oxalocrotonate tautomerase (4-OT), which belongs to the same family of enzymes as CaaD,[4b] exhibits promiscuous dehalogenase activity towards **4** that can be slightly increased (~9-fold in terms of k_{cat}) by the replacement of the active site residue Leu-8 with an arginine.[10a] This observation prompted us to test whether mutations at the corresponding position in mouse MIF, which belongs to a distinct family within the tautomerase superfamily,[4b] may also affect the promiscuous dehalogenase activity of this cytokine. A superpositioning of the crystal structure of 4-OT (PDB 1BJP)[14] with that of mouse MIF in complex with (*E*)-2-fluoro-*p*-hydroxycinnamate (PDB 1MFI)[15], a competitive inhibitor of MIF's PPT activity, showed that Ile-64 in MIF is structurally homologous to Leu-8 in 4-OT. Therefore, Ile-64 was randomized using a site-saturation mutagenesis (SSM) strategy. The SSM library was used to transform *E. coli* BL21(DE3) cells, and ~400 transformants were evaluated for enhanced dehalogenase activity. Unfortunately, the MIF mutants in this library showed no improved activity towards **4**.

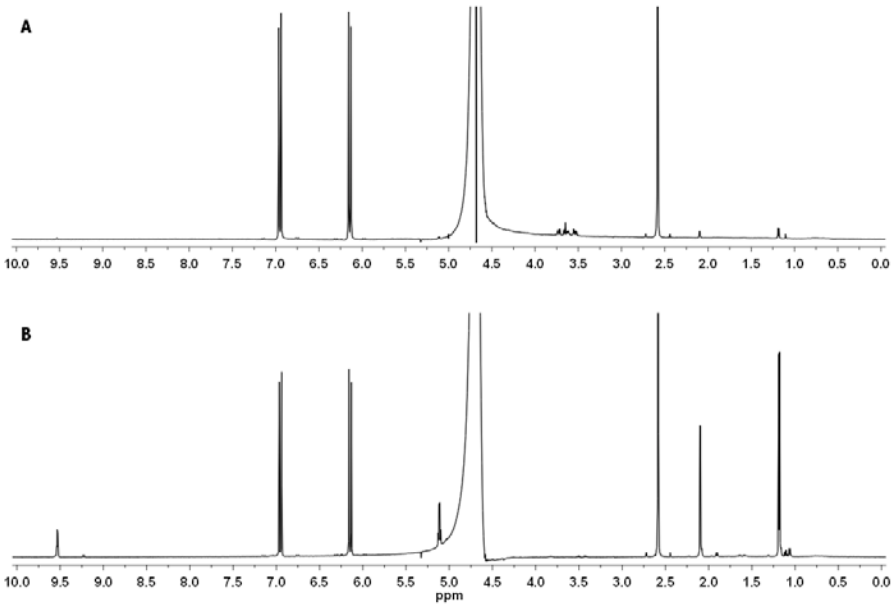


Figure 1. ^{1}H NMR spectra monitoring the dehalogenation of *trans*-3-chloroacrylate (**4**) catalyzed by wild-type MIF (A) and MIF mutant I64V/V106L (B) in 100 mM phosphate buffer at pH 6.5. Spectra were recorded after 7 days of incubation at 22°C. The signals at 6.15 and 6.96 ppm correspond to **4**, the signals at 2.10 and 9.53 ppm correspond to acetaldehyde, and the signals at 1.18 and 5.11 ppm correspond to the hydrate of acetaldehyde. The signal at 2.58 ppm corresponds to DMSO.

We then applied a multiple-site saturation strategy, in which position Ile-64 was randomized in combination with another active site position (or positions) (Figure 2). Four double-site saturation mutagenesis libraries (I64X/I67X, I64X/K32X, I64X/Y36X, I64X/W108X) and one triple-site saturation mutagenesis library (I64X/V106X/F113X) were constructed. Each double-site library was screened by evaluating about 2000 transformants, while approximately 4000 transformants were evaluated for the triple-site library. Whereas the mutants in the double-site libraries showed no improved dehalogenase activity, one mutant with strongly enhanced dehalogenase activity towards **4** was identified in the triple-site library. DNA sequencing revealed that this MIF mutant carries two mutations, namely I64V and V106L.

The I64V/V106L mutant was overexpressed, purified to homogeneity, and incubated with **4** in 100 mM phosphate buffer at pH 6.5 and 22°C. The reaction was monitored by ^{1}H NMR spectroscopy. Integration of the signals indicates that ~42% of **4** has been converted to acetaldehyde and its hydrate after 7 days (Figure 1B). After we established that the mutant enzyme shows improved dehalogenase activity, kinetic parameters were determined. The I64V/V106L mutant catalyzes the dehalogenation of **4** with a k_{cat} = 6×10^{-3} s^{-1} and a K_m of 18.2 mM, which results in a k_{cat}/K_m of 3.3×10^{-1} M^{-1} s^{-1}. A comparison of this k_{cat} value to that measured for wild-type MIF (Table 1), shows that

the I64V/V106L mutant has a 200-fold improved dehalogenase activity towards **4**. This MIF mutant catalyzes the tautomerization of **1** to **2** (the original activity) with a k_{cat} = 14 s^{-1}, which is only 8-fold lower than the k_{cat} value determined for the same reaction catalyzed by wild-type MIF (Table 1).

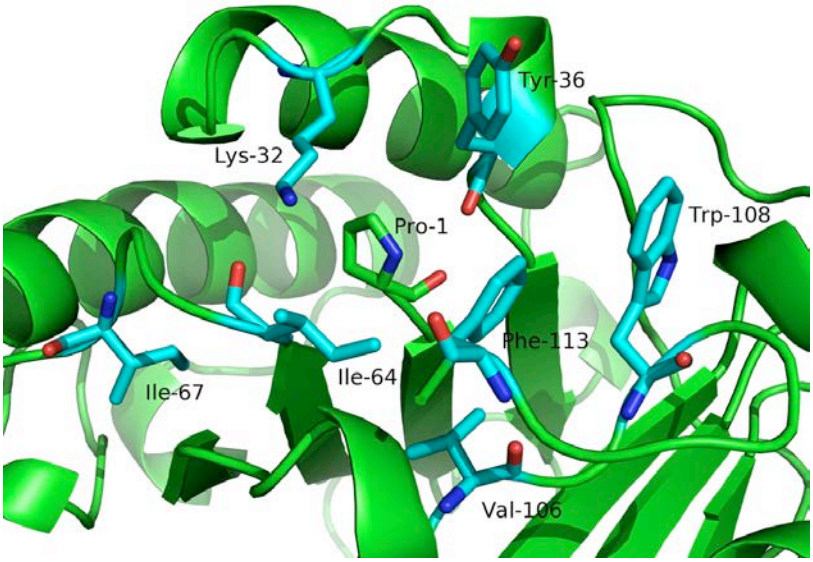


Figure 2. A close-up of the active site of MIF.[15] For clarity, the atoms are colored as follows: red, oxygens; blue, nitrogens; green, carbons of Pro-1 of MIF; cyan, carbons of residues targeted in the saturation mutagenesis approach.

The active site residues Pro-1 and Lys-32 are important for the native PPT activity of MIF. Pro-1 acts as the catalytic base, abstracting the hydroxyl proton of **1** for delivery to the C-3 position (yielding **2**), and Lys-32 plays a role in substrate binding by interacting with the carboxylate group of **1**.[5] To evaluate the importance of Pro-1 and Lys-32 for the promiscuous CaaD activity of mutant I64V/V106L, the corresponding alanine mutants were prepared. In separate reactions, the P1A/I64V/V106L and K32A/I64V/V106L mutants were incubated with **4** in 100 mM phosphate buffer at pH 6.5 and at 22°C, and the two reactions were monitored by ^{1}H NMR spectroscopy. The incubation with the P1A/I64V/V106L mutant showed no product formation after 7 days, while the incubation with the K32A/I64V/V106L mutant showed 35% conversion of **4** into a mixture of acetaldehyde and its hydrate after 7 days. These results suggest that Pro-1 is critical for the CaaD activity of mutant I64V/V106L, whereas Lys-32 appears not to be important for the dehalogenase activity of this mutant. The importance of Pro-1 to the CaaD activity of MIF (I64V/V106L), indicates that dehalogenation, like tautomerization, is an active site process that involves Pro-1.[16]

Table 1. Kinetic parameters for MIF and MIF mutant I64V/V106L using phenylenolpyruvate (**1**) and *trans*-3-chloroacrylic acid (**4**).

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
MIF WT[a]	**1**	114 ± 13	0.50 ± 0.12	2.3×10^5
MIF WT[b]	**4**	3×10^{-5}	5.8 ± 1.2	5×10^{-3}
I64V/V106L MIF[a]	**1**	14 ± 3	0.38 ± 0.13	3.6×10^4
I64V/V106L MIF[b,c]	**4**	6×10^{-3}	18.2 ± 4	3.3×10^{-1}

[a]These steady-state kinetic parameters were measured in 20 mM Na_2HPO_4 buffer (pH 7.5) at 22°C. [b]These kinetic data were measured in 50 mM Tris-SO_4 buffer (pH 6.5) at 22°C. [c] Substrate inhibition was observed at high concentrations of **4** (apparent K_i of ~64 mM).

In summary, we have shown that mouse MIF has low-level CaaD activity, which can be improved 200-fold (in terms of k_{cat}) by two mutations (I64V/V106L) in the substrate binding pocket of this cytokine. The active site Pro-1 residue of MIF is critical for this reaction because the P1A/I64V/V106L mutant has no detectable dehalogenase activity. Although the CaaD activity of the engineered MIF variant I64V/V106L is still low, the rates of dehalogenation are significant in comparison with the reported nonenzymatic rate of ~2.2×10^{-12} s^{-1} at 25 °C and pH 7.[17] Using this value for the uncatalyzed dehalogenation of **4** and the k_{cat} value for the MIF (I64V/V106L)-catalyzed dehalogenation of **4** (Table 1), it can be estimated that this mutant cytokine affords an ~10^9-fold rate enhancement. This rate enhancement (k_{cat}/k_{uncat}) matches the rate enhancements afforded by many conventional enzymes that act on their natural substrates.[17] Hence, these results show that the evolution of dehalogenase activity towards a xenobiotic organohalogen can be surprisingly facile, requiring only a few mutations in the active site of a cytokine.

The dehalogenase activity of MIF is a fascinating example of a promiscuous activity that has been discovered by looking for a specific reaction based on a protein's relatives. Indeed, promiscuous activities could provide important clues regarding the progenitor of a diverged (super)family member.[18] The shared dehalogenase and tautomerase activities of mouse MIF and bacterial enzymes of the tautomerase superfamily provide evidence for divergent evolution from a common ancestral enzyme, and strongly support the hypothesis that the enzymatic activities of MIF are vestigial in mammals.[11] Given that MIF was presumably recruited long ago to serve a non-enzymatic function, it is quite remarkable that it still exhibits promiscuous enzymatic activities typical of other superfamily members. Directed evolution experiments to further increase the CaaD activity of MIF are underway, with the aim to identify additional features necessary for converting this cytokine into a fully active dehalogenating enzyme.

References

1 a) B. R. Bloom, B. Bennett, *Science* **1996**, *153*, 80-82; b) J. R. David, *Proc. Natl. Acad. Sci. USA* **1966**, *56*, 72-77; c) H. Lue, R. Kleemann, T. Calandra, T. Roger, J. Bernhagen, *Microbes Infect.* **2002**, *4*, 449-460; d) R. Bucala, *Nature* **2000**, *408*, 146-147; e) J. Bernhagen, R. A. Mitchell, T. Calandra, W. Voelter, A. Cerami, R. Bucala, *Biochemistry* **1994**, *33*, 14144-14155; f) S. C. Donnelly, C. Haslett, P. T. Reid, I. S. Grant, W. A. H. Wallace, C. N. Metz, L. J. Bruce, R. Bucala, *Nature Medicine* **1997**, *3*, 320-323; g) A. Mikulowska, C. N. Metz, R. Bucala, R. Holmdahl, *J. Immunol*. **1997**, *158*, 5514-5517; h) E. F. Morand, M. Leech, J. Bernhagen, *Nat. Rev. Drug Discov.* **2006**, *5*, 399-410; i) T. Calandra, T. Roger, *Nat. Rev. Immunol.* **2003**, *3*, 791-800; j) T. Calandra, J. Bernhagen, C. N. Metz, L. A. Spiegel, M. Bacher, T. Donnelly, A. Cerami, R. Bucala, *Nature* **1995**, *377*, 68-71.

2 E. Rosengren, P. Aman, S. Thelin, C. Hansson, S. Ahlfors, P. Bjork, L. Jacobsson, H. Rorsman, *FEBS Lett.* **1997**, *417*, 85-88.

3 a) M. Suzuki, H. Sugimoto, A. Nakagawa, I. Tanaka, J. Nishihira, M. Sakai, *Nat. Struct. Biol.* **1996**, *3*, 259-266; b) H. -W. Sun, J. Bernhagen, R. Bucala, E. Lolis, *Proc. Natl. Acad. Sci.* **1996**, *93*, 5191-5196.

4 a) A. G. Murzin, *Curr. Opin. Struct. Biol*. **1996**, *6*, 386-394; b) G. J. Poelarends, V. Puthan Veetil, C. P. Whitman, **Cell. Mol. Life Sci**. **2008**, *65*, 3606-3618.

5 a) S. L. Stamps, M. C. Fitzgerald, C. P. Whitman, *Biochemistry* **1998**, *37*, 10195-10202; b) J. B. Lubetsky, M. Swope, C. Dealwis, P. Blake, E. Lolis, *Biochemistry* **1999**, *38*, 7346-7354; c) S. L. Stamps, A. B. Taylor, S. C. Wang, M. L. Hackert, C. P. Whitman, *Biochemistry* **2000**, *39*, 9671-9678.

6 a) G. J. Poelarends, R. Saunier, D. B. Janssen, *J. Bacteriol.* **2001**, *183*, 4269–4277; b) S. C. Wang, M. D. Person, W. H. Johnson, Jr., C. P. Whitman, *Biochemistry* **2003**, *42*, 8762–8773; c) R. M. de Jong, W. Brugman, G. J. Poelarends, C. P. Whitman, B. W. Dijkstra, *J. Biol. Chem.* **2004**, *279*, 11546–11552.

7 a) G. J. Poelarends, H. Serrano, M. D. Person, W. H. Johnson, Jr., A. G. Murzin, C. P. Whitman, *Biochemistry* **2004**, *43*, 759–772; b) R. M. de Jong, P. Bazzacco, G. J. Poelarends, W. H. Johnson, Jr., Y. –J. Kim, E. A. Burks, H. Serrano, A. –M. W. H. Thunnissen, C. P. Whitman, B. W. Dijkstra, *J. Biol. Chem.* **2007**, *282*, 2440–2449.

8 a) G. J. Poelarends, M. Wilkens, M. J. Larkin, J. D. van Elsas, D. B. Janssen, *Appl. Environ. Microbiol.* **1998**, *64*, 2931–2936; b) G. J. Poelarends, C. P. Whitman, *Bioorg. Chem.* **2004**, *32*, 376–392.

9 a) G. J. Poelarends, H. Serrano, W. H. Johnson, Jr., C. P. Whitman, *Biochemistry* **2007**, *46*, 9596-9604; b) G. J. Poelarends, C. P. Whitman, in *Comprehensive Natural Products II: Chemistry and Biology* (Mander, L.N., and Liu, H.-W., Eds.) **2010**, volume 8, pp 89-123, Elsevier, Oxford, UK.

10 a) G. J. Poelarends, J. J. Almrud, H. Serrano, J. E. Darty, W. H. Johnson, Jr., M. L. Hackert, C. P. Whitman, *Biochemistry* **2006**, *45*, 7700-7708; b) B.-J. Baas, E. Zandvoort, A. A. Wasiel, W. J. Quax, G. J. Poelarends, *Biochemistry* **2011**, *50*, 2889-2899.

11 a) A. Sato, T. S. Uinuk-ool, N. Kuroda, W. E. Mayer, N. Takezaki, R. Dongak, F. Figueroa, M. D. Cooper, J. Klein, *Developmental and Comparative Immunology* **2003**, *27*, 401-412; b) A. A. Wasiel, H. J. Rozeboom, D. Hauke, B.-J. Baas, E. Zandvoort, W. J. Quax, A.-M. W. H. Thunnissen, G. J. Poelarends, *Biochemistry* **2010**, *49*, 7572-7581.

12 A likely scenario for the formation of acetaldehyde involves the initial hydrolytic dehalogenation of **4** to yield malonate semialdehyde (**6**, Scheme 2), which then undergoes decarboxylation to yield acetaldehyde. The absence of ^{1}H NMR signals for **6** suggests either that **6** is not sufficiently stable to accumulate in quantities detectable by ^{1}H NMR spectroscopy or MIF catalyzes the decarboxylation of **6** to yield acetaldehyde.

13 a) S. Keuning, D. B. Janssen, B. Whitholt, *J. Bacteriol.* **1985**, *163,* 635-639; b) B.-J. Baas, E. Zandvoort, A. A. Wasiel, W. J. Quax, G. J. Poelarends, *Biochemistry* **2011**, *50*, 2889-2899.

14 A. B. Taylor, R. M. Czerwinski, W. H. Johnson, Jr., C. P. Whitman, M. L. Hackert, *Biochemistry* **1998**, *37*, 14692-14700.

15 A. B. Taylor, W. H. Johnson, Jr., R. M. Czerwinski, H. –S. Li, M. L. Hackert, C. P. Whitman, *Biochemistry* **1999**, *38*, 7444-7452.

16 The mechanistic roles of Pro-1 and other active site residues in the promiscuous CaaD activity of mouse MIF (wildtype or mutant I64V/V106L) are currently not known.

17 C. M. Horvat, R. V. Wolfenden, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 16199-16202.

18 a) L. Afriat, C. Roodveldt, G. Manco, D. S. Tawfik, *Biochemistry* **2006**, *45*, 13677-13686; b) O. Khersonsky, D. S. Tawfik, *Annu. Rev. Biochem.* **2010**, *79*, 471-505.

Author contributions

A.A.W. designed and performed most of the experiments, and analyzed the data. B.-J.B. developed the mutation strategy and screening assay. E.Z. preformed the ^{1}H NMR experiments and interpreted the data. W.J.Q. and G.J.P. directed all aspects of the project. A.W. and G.J.P. wrote the manuscript. All authors gave feedback on the manuscript.

Supplementary information.

Supplementary Materials and Methods

Materials. All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless stated otherwise. The sources of the biochemicals, buffers, solvents, components of Luria-Bertani (LB) media as well as the materials, enzymes, and reagents used in the molecular biology procedures are reported elsewhere.[1]

General methods. Procedures for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were performed as described elsewhere.[2] The PCR was carried out in a DNA thermal cycler (model GS-1) obtained from Biolegio (Nijmegen, The Netherlands). DNA sequencing was performed by Macrogen (Seoul, Korea). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 10% polyacrylamide. The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the Waddell method.[3] Kinetic data were obtained on a V-650 spectrophotometer from Jasco (IJsselstein, The Netherlands). The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus, Software Ltd., Horley, U.K.) or Sigma Plot 11.0 (Systat Software Inc., Chicago, USA). ^{1}H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale (δ scale) and are referenced to protium (H_2O: δ = 4.67). The masses of MIF and MIF mutants were determined by ESI-MS using a Sciex API 3000 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario, Canada), housed in the Mass Spectrometry Facility Core in the Department of Pharmacy at the University of Groningen.

Construction of site-saturation mutagenesis libraries of MIF. One single-site saturation mutagenesis library (I64X), four double-site saturation mutagenesis libraries (I64X/I67X, I64X/K32X, I64X/Y36X, I64X/W108X), and one triple-site saturation mutagenesis library (I64X/V106X/F113X) were generated by the overlap extension PCR method[4] using plasmid pET20b (MIF-His)[1] as a template. For the construction of the I64X library, the following primers were used: MIF forward primer (5'-CAG CGA **CAT ATG** CCT ATG TTC ATC-3'; *Nde*I restriction site in bold), MIF-I64Rw (5'- CC GAT CTT GCC SNN GCT GTG CAG GCT-3'), MIF-I64Fw (5'-AGC CTG CAC AGC NNS GGC AAG ATC GGT GGT GCC-3'), and MIF reverse primer (5'-CTG ATG GAT **CTC GAG** AGC GAA GGT GGA ACC-3'; *Xho*I restriction site in bold). For the construction of the I64X/W108X library, the following primers were used: MIF forward primer, MIF-I64Rw, MIF-I64Fw, MIF-W108Rw (5'-CT GAT GGA TCC TCA AGC GAA GGT GGA ACC GTT SNN GCC CAC GTT GGC AGC GTT CAT-3'), and MIF reverse primer. For the construction of the I64X/I67X library, the following primers were used: MIF forward primer, MIF-I64/I67Rw (5'-GTT CTG GGC ACC ACC SNN CTT GCC SNN GCT GTG CAG GCT GCA GAG-3'), MIF-I64/I67Fw (5'-CTC TGC AGC CTG CAC AGC NNS GGC AAG NNS GGT GGT GCC CAG AAC-3'), and MIF reverse primer. For the construction of the I64X/K32X library, the following primers were used: MIF forward primer, MIF-K32Rw (5'-GAT GTA CTG TGC GGG SNN GCC GGT GGC CTG CGC-3'), MIF-K32Fw (5'-GCG CAG GCC

ACC GGC NNS CCC GCA CAG TAC ATC-3'), MIF-I64Rw, MIF-I64Fw, and MIF reverse primer. For the construction of the I64X/Y36X library, the following primers were used: MIF forward primer, MIF-Y36Rw (5'-CAC GTG CAC TGC GAT SNN CTG TGC GGG CTT GCC-3'), MIF-Y36Fw (5'-GGC AAG CCC GCA CAG NNS ATC GCA GTG CAC GTG-3'), MIF-I64Rw, MIF-I64Fw, and MIF reverse primer. For the construction of the I64X/V106X/F113X library, the following primers were used: MIF forward primer, MIF-I64Rw, MIF-I64Fw, MIF-V016/F113Rw (5'-CT GAT GGA TCC TCA AGC SNN GGT GGA ACC GTT CCA GCC SNN GTT GGC AGC GTT CAT-3'), and MIF reverse primer.

The final full-length PCR products were gel purified. Subsequently, the PCR products and the pET20b(+) vector (Novagen) were digested with *Nde*I and *Xho*I restriction enzymes, purified, and ligated using T4 DNA ligase. An aliquot of each ligation mixture was transformed into competent *E. coli* DH10B cells. Transformants were selected at 37°C on LB plates containing ampicillin (100 μg/ml). For each library, plasmid DNA was isolated from several colonies and analyzed for the presence of the insert by *NdeI* and *XhoI* digestion. The cloned genes were sequenced to verify that the desired codons had been mutated during the amplification of the genes.

Construction of the P1A/I64V/V106L and K32A/I64V/V106L mutants. The P1A and K32A mutants of MIF-I64V/V106L were generated by the overlap extension PCR method[4] using plasmid pET20b(I64V/V106L-MIF-His) (this study) as the template. To introduce the P1A mutation, the following forward primer was used: 5'-CAG CGA **CAT ATG** GCT ATG TTC ATC GTG-3', with the *Nde*I site in bold, in combination with the MIF reverse primer. For the introduction of the K32A mutation, the following primers were used: MIF forward primer, reverse primer 5'-GAT GTA CTG TGC GGG AGC GCC GGT GGC CTG CGC-3', forward primer 5'-GCG CAG GCC ACC GGC GCT CCC GCA CAG TAC ATC-3', and MIF reverse primer. The final PCR products were cloned in the expression vector pET20b(+) as described above. The mutant genes were completely sequenced (with overlapping reads) to verify that only the intended mutation had been introduced.

Expression and purification of His-tagged MIF proteins. The MIF proteins, either wild-type or mutant, were overexpressed in *E. coli* BL21(DE3) and purified to homogeneity as described before[1] with the following modification. All buffers were prepared without NaCl to minimize chloride contamination, which would interfere with the colorimetric assay that monitors chloride release.[5,6] The subunit mass of each purified MIF protein was determined by ESI-MS to verify the initiating methionine had been removed during posttranslational processing, resulting in a protein with an N-terminal proline. The purified proteins were stored at +4 °C or −80 °C until further use.

Enzymatic Assays. The ketonization of phenylenolpyruvate (**1**) by MIF and the I64V/V106L mutant was monitored by following the depletion of **1** at 255 nm (ε = 6615 M^{-1} cm^{-1}) in 20 mM NaH_2PO_4 buffer at pH 6.5 and at 22°C as decribed before.[1] The dehalogenation activities of MIF and MIF mutants were measured by following the dechlorination of *trans*-3-chloroacrylate (**4**) at 22°C in 50 mM Tris-SO_4 buffer (pH 6.5) using a colorimetric assay.[5,6] An appropriate amount of enzyme was incubated with the desired concentration of **4** in 3 mL of the Tris-SO_4 buffer. Stock solutions of **4** were

made up in 50 mM Tris-SO_4 buffer, and the pH was adjusted to 6.5. Chloride concentrations were measured colorimetrically at different time intervals.

Screening mutant libraries for MIF variants with enhanced dehalogenase activity. To screen MIF mutants for dehalogenase activity, each mutant DNA library was transformed into *E. coli* BL21(DE3) cells, and transformants were selected on LB/ampicillin agar plates without NaCl. Single colonies were picked and transferred to a new (numbered) LB/ampicillin agar plate without NaCl, and incubated overnight at 37°C. Subsequently, a small amount of cells from each colony was incubated in a 96-wells microtiter plate with 100 µL of a mixture of 50 mM *cis*- (**5**) and *trans*-3-chloroacrylic acid (**4**) in Tris-SO_4 buffer (pH 7.5). The plates were covered with a plastic sheet and incubated at 37°C for 48 hours. After the incubation period, 70 µL of 0.25 M $NH_4Fe(SO_4)_2$ in 6 M HNO_3 followed by 30 µL of saturated $Hg(SCN)_2$ in ethanol were added. A red colour indicated the presence of dehalogenase activity. By comparing the intensity of the red colour between cells expressing MIF mutants and those expressing wild-type MIF (*E. coli* BL21-DE3 harboring pET20b-MIF), one MIF mutant with strongly enhanced dehalogenase activity could be identified. Control experiments with purified mutant enzyme showed that this MIF variant only dehalogenates **4** but displays no activity towards **5**.

^{1}H NMR spectroscopic analysis of the dehalogenation reaction. A series of ^{1}H NMR spectra monitoring the dehalogenation of *trans*-3-chloroacrylic acid (**4**) or *cis*-3-chloroacrylic acid (**5**) catalyzed by wildtype MIF or MIF mutants were recorded according to protocols described elsewhere.[6]

Supplementary references

1 A. A. Wasiel, H. J. Rozeboom, D. Hauke, B.-J. Baas, E. Zandvoort, W. J. Quax, A.-M. W. H. Thunnissen, G. J. Poelarends, *Biochemistry* **2010**, *49*, 7572-7581.

2 J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, **1989**.

3 W. J. Waddell, *J. Lab. Clin. Med.* **1956***, 48*, 311-314.

4 S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene*, **1989**, *77*, 51-59.

5 S. Keuning, D. B. Janssen, B. Whitholt, *J. Bacteriol.* **1985**, *163,* 635-639.

6 B.-J. Baas, E. Zandvoort, A. A. Wasiel, W. J. Quax, G. J. Poelarends, *Biochemistry* **2011**, *50*, 2889-2899.