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Identification of a Novel Determinant of Glutathione Affinity in Dichloromethane Dehalogenases/Glutathione *S*-Transferases

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Bacterial dichloromethane dehalogenases catalyze the glutathione-dependent hydrolysis of dichloromethane to formaldehyde and are members of the enzyme superfamily of glutathione *S*-transferases involved in the detoxification of electrophilic compounds. Numerous protein engineering studies have addressed questions pertaining to the substrate specificity, the reaction mechanism, and the kinetic pathway of glutathione *S*-transferases. In contrast, the molecular determinants for binding of the glutathione cofactor have been less well investigated. Dichloromethane dehalogenases from *Hyphomicrobium* sp. DM2 and *Methylobacterium* sp. DM4 displayed significantly different affinities for glutathione, but not for the dichloromethane substrate. The sequence of *dcmA*, the dichloromethane dehalogenase gene from strain DM2, was determined and featured a single base difference from the previously determined sequence of *dcmA* from strain DM4. This base change resulted in a single amino acid difference in the corresponding proteins at sequence position 27. Site-directed variants of the homologous dichloromethane dehalogenase from *Methylophilus* sp. DM11 (56% amino acid identity) at the corresponding residue in the protein sequence provided further evidence that this residue selectively modulated the dependence of dichloromethane dehalogenase activity on glutathione. © 1997 Academic Press

Key Words: **dichloromethane dehalogenase; glutathione *S*-transferase; glutathione.**

1. INTRODUCTION

Dichloromethane dehalogenases (EC 4.5.1.3) catalyze the glutathione-dependent transformation of dichloromethane to formaldehyde (see (1) for a review). Bacteria possessing this enzyme are able to grow on dichloromethane as the sole source of carbon and energy for growth. The gene encoding dichloromethane dehalogenase (*dcmA*) was cloned and sequenced from *Methylobacterium* sp. DM4 (2) and *Methylophilus* sp. DM11 (3). Both functionally and by their sequence, these enzymes belong to the enzyme superfamily of glutathione *S*-transferases (GST; EC 2.5.1.18; see (4) for a review). Few representatives of glutathione *S*-transferases are yet known in bacteria. Only sequence data are available in most cases, although results with bacterial enzymes for which biochemical investigations have been carried out suggest enzymatic functions not usually observed in eukaryotic GST (5). Bacterial dichloromethane dehalogenases have a number of distinctive features compared to most GST. First, glutathione conjugates generated in the enzymatic reaction are unstable and are hydrolyzed to glutathione, formaldehyde and hydrochloric acid. Second, the substrate range of dichloromethane dehalogenases is essentially restricted to dihalomethanes, and typical model GST substrates such as aromatic chlorinated compounds, peroxides, or epoxides are not turned over by these enzymes (S.V., unpublished data). Finally, dichloromethane dehalogenases are not retained on glutathione-derivatized chromatography supports used in the affinity purification of GST enzymes. Previous protein engineering studies have addressed the importance of several amino acid residues in the catalytic efficiency and substrate specificity of the dichloromethane dehalogenase from *Methylophilus* sp. DM11 (6). We report here on further studies which have allowed us to uncover a position in the protein sequence of dichloromethane dehalogenases/glutathione *S*-transferases which specifically modulates the affinity of these enzymes for the glutathione cofactor.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals were of the highest purity available and were purchased from Fluka. Formaldehyde dehydrogenase was

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Abbreviations used: DCM, dichloromethane; DIG, digoxigenin; GST, glutathione *S*-transferase.

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obtained form Sigma, oligonucleotides from Microsynth, restriction enzymes from Fermentas, Boehringer, or New England Biolabs, and Taq polymerase from Fermentas.

2.2. Bacterial strains. Bacterials strains used were *Methylobacterium* sp. strain DM4 (7), *Hyphomicrobium* sp. strain DM2 (8), *Methylophilus* sp. strain DM11 (9), *E. coli* strains DH5α for recombinant DNA work, and *E. coli* BL21(DE3)pLysS for protein expression.

2.3. Site-directed mutagenesis. Mutations were introduced in the *dcmA* gene encoding the dichloromethane dehalogenase from strain DM11 by PCR using the megaprimer method (10) as described previously (6). Briefly, mutations in codon 18 of the *dcmA* gene were generated by amplification of a fragment of the *dcmA* gene with the degenerate forward primer TCGTGCA**RY**TCATCAATTTATGC (pos. 48-70) and the reverse primer CAATGAAATGCACCGCC (pos. 353-337) using the mutagenesis and expression plasmid pME1919 (6) encoding the wild-type *dcmA* gene as the template. Base mixtures of A:G (2.1 ratio) at position R and C:T (2:1 ratio) at position Y in the mutagenic olignucleotide allowed for the generation of mutant codons ACT (Thr), GCT (Ala), ATT (Ile), as well as the wild-type GTT (Val) codon at this location in the *dcmA* gene. The obtained 306 bp PCR product was gel-purified and used as the megaprimer in a second PCR on the same template DNA with the universal T7 primer AATACGACTCACTATAGG located upstream of the *dcmA* gene in the expression plasmid. The obtained 432 bp PCR product was digested with *Xba*I and *Nsi*I and the resulting 349 bp fragment exchanged against the corresponding fragment in the expression plasmid containing the wild-type *dcmA* gene.

2.4. DNA sequencing. DNA sequencing was performed by PCR methods using digoxigenin (DIG) labelled primers and the GATC membrane blotting system (11). The sequence of the *dcmA* gene from strain DM2 was determined from plasmids obtained in the course of restriction analysis of the *dcm* region of strains DM2 and DM4 ((12) and unpublished data), with oligonucleotide primers previously used in the sequence determination of the *dcmA* gene from strain DM4 (2). The PCR-generated mutagenized fragments in mutant clones of the DM11 *dcmA* gene were sequenced to determine the nature of the base change at codon 18, and to ensure that no additional mutations had been introduced during PCR amplification.

2.5. Protein expression and purification. Strains DM2 and DM4 were grown at 30°C in minimal medium with a mixture of 10 mM each of methanol and dichloromethane as the carbon source (7). *E. coli* BL21(DE3)pLysS cells transformed with expression plasmid pME1919 or its mutagenized derivatives were grown in LB medium at 18°C containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) as described previously (6). Dichloromethane dehalogenases were purified to greater than 90% homogeneity by ammonium sulfate fractionation followed by anion exchange (6), except that purification of the DCM dehalogenases from strains DM2 and DM4 was performed at pH 8.0 instead of pH 7.5.

2.6. Enzyme activity measurements. Steady state rates of DCM dehalogenase activity were determined spectrophotometrically using a coupled enzyme assay with formaldehyde dehydrogenase (6). Enzymes were assayed near their pH optimum (pH 8.0 for the DM2 and DM4 enzymes, and pH 8.5 for the wild-type DM11 enzyme and its mutants). The concentration of glutathione in the determinations of enzyme activity as a function of dichloromethane concentration was 2-5 mM for the DM2 and DM4 enzymes, and 1 mM for the DM11 enzyme. The data obtained were fitted to the Michaelis-Menten equation ($v = k_{cat}\,[E]_t[S]/\{K_m + [S]\}$) or the Hill equation ($v = k_{cat}\,[E]_t/\{1 + (S_{0.5} + [S])^{n_H}\}$) by non-linear least-square fitting using Kaleidagraph (Abelbeck Software) as reported previously (6). Standard errors amounted to less than 20% of the K_m or $S_{0.5}$ value, and to less than 10% of the k_{cat} and n_H values, respectively.

3. RESULTS

We are currently investigating the sequence variation of *dcmA*, the structural gene of dichloromethane dehalogenase, among independently isolated dichloromethane utilizing methylotrophic bacteria. In the course of this analysis, a single base difference in the first base of codon 27 of the *dcmA* gene was detected between the previously sequenced gene from strain DM4 (ACA) and that from strain DM2 (GCA). This base substitution resulted in sequence variation between the two enzymes from strains DM2 (Thr) and DM4 (Ala) at a single position of the protein sequence (Fig. 1). The enzymatic properties of the two enzymes had already been reported earlier (8,9), and were newly determined in the light of the sequencing results (Table 1). The two enzymes significantly differed in their affinity for the glutathione cofactor, but not in their affinity for the substrate dichloromethane, indicating a role in glutathione binding for the residue at position 27 dichloromethane dehalogenases from strains DM2 and DM4. Attempts to express the strain DM4 dichloromethane dehalogenase in active form in *E. coli* were unsuccessful. We therefore investigated by site-directed mutagenesis the effect of substitutions at Val18, the homologous residue of the enzyme from strain DM11 (Fig. 1), on its kinetic properties. The DM11 enzyme shows only 56% sequence identity to the enzymes from strains DM2 and DM4 (3), and features a higher turnover number, a lower affinity for dichloromethane, and a higher affinity for glutathione. As shown in Table 1, the nature of the residue at position 18 in dichloromethane dehalogenases from strain DM11 indeed modulated glutathione affinity. In contrast, the affinity of the enzyme for dichloromethane and its discrimination between dichloromethane and dibromomethane were not significantly affected in mutant enzymes.

The dependence of the turnover rate on the concentration of the glutathione cofactor differs between the enzymes from strains DM2 and DM4 and the enzyme from strain DM11 (6,9). DM2 and DM4 DCM dehalogenases, as all GSTs described so far, display a hyperbolic dependence of enzyme activity on glutathione concentration. In contrast, the DM11 enzyme displays a sigmoidal dependence. Reciprocal plots of substrate concentration versus catalytic rate readily reveal such behaviour through their departure from the linearity expected for Michaelis-Menten kinetics (Fig. 2; (6)). We found that the concentration of dichloromethane in the assay mixture had a striking effect on the sigmoidicity of the dependence of the catalytic rate on glutathione in the wild-type DM11 enzyme, as seen from the observed reduction in the Hill coefficient n_H (Table 2), and the reduced departure from linearity in double-reciprocal plots of glutathione concentration versus activity (Fig. 2). At saturating concentrations of substrate (1 mM), the observed rate of formaldehyde formation displayed a sigmoid dependence on the glutathione concentration. At 20 μM, a substrate concentration below the K_m for dichloromethane, this sigmoidicity was much reduced in the wild type enzyme. This effect was en-

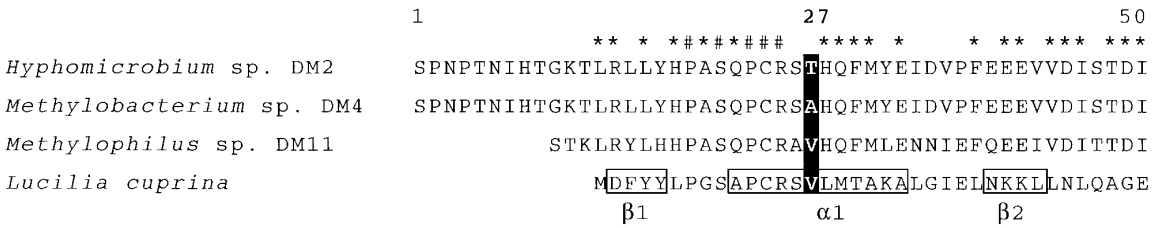

FIG. 1. Alignment of dichloromethane dehalogenase N-terminal sequences with the most closely related sequence from a GST of known structure, that from *Lucilia cuprina* (13). Elements of secondary structure of the *Lucilia* enzyme are boxed. Dichloromethane dehalogenase sequences from strains DM2 and DM4 differ only at position 27 (shown white on black). Residues identical between dichloromethane dehalogenases are indicated by an asterisk, and those identical in all sequences are indicated by a hash.

hanced in DM11 DCM dehalogenase protein mutants (Table 2). A reduced sigmoidicity of the dependence of enzyme activity on glutathione was already observed at saturating concentrations of dichloromethane, and this effect increased at a low dichloromethane concentration (Table 2). In contrast, no significant trend was observed for the effect of dichloromethane concentration on the glutathione affinity of the proteins *per se*.

4. DISCUSSION

The importance for glutathione binding of residue 27 of DCM dehalogenases from strain DM2 and DM4, and of the corresponding residue 18 of the homologous enzyme from strain DM11, is quite surprising. No X-ray structure of a dichloromethane dehalogenase enzyme is yet available, but alignments of dichloromethane dehalogenase sequences with that of the nearest GST enzyme of known structure from the insect *Lucilia cuprina* (13), and molecular modelling studies of the DCM dehalogenase from strain DM11 (14), suggest a location of this residue near the middle of the first helix in the canonical GST fold. In the *Lucilia* enzyme, the corresponding valine residue is not within bonding distance (d = 8-12 Å) of the glutathione cofactor. We speculate that amino acid changes at this location in dichloromethane dehalogenases, and possibly also in other GST enzymes of the Theta-class, cause conformational changes in the interactions of helix 1 with the rest of the protein that significantly influence glutathione binding. Indeed, the modes of glutathione binding differ significantly among GST enzymes of the Theta-class. Published values of glutathione affinity vary widely, ranging from 40 μM in *E. coli* (15) and 66 μM in *Methylophilus* sp. DM11 (6), to 510 μM in the *Lucilia cuprina* enzyme (16), and 686 μM in the GST from *Proteus mirabilis* (17). These numbers do not correlate with the binding behaviour of GST enzymes to glutathione-derivatized supports used for affinity purification, since the *E. coli*, *P. mirabilis* and *L. cuprina* enzymes, but not the dichloromethane dehalogenases, are retained on such supports.

The reduction of sigmoid behaviour in glutathione binding upon mutation of the DM11 dichloromethane dehalogenase is suggestive of an enzymatic reaction pathway by which glutathione binding prior to dichloromethane binding speeds up the steady-state kinetics of the wild-type enzyme, and amino acid variations at position 18 cause a reduction in this binding preference. It remains to be determined how such binding events, possibly involving conformational changes in the protein, are able to affect the rather slow rates observed for the overall enzyme reaction. It is noteworthy that sigmoid enzyme kinetics do not necessarily

TABLE 1

Kinetic Properties of Dichloromethane Dehalogenases

Enzyme	Residue[a]	K^{GSH} (μM)	K_m^{DCM} (μM)	k_{cat} (s^{-1})	$k_{cat}^{DBM}/k_{cat}^{DCM}$
Hyphomicrobium sp. DM2	Thr27	174[b]	11	0.6	1.0
Methylobacterium sp. DM4	Ala27	403[b]	9	0.6	0.9
Methylophilus sp. DM11 wild-type	Val18	66[c]	59	3.3	1.8
Methylophilus sp. DM11 V18T	Thr18	91[c]	60	2.2	1.5
Methylophilus sp. DM11 V18A	Ala18	112[c]	41	2.2	1.6
Methylophilus sp. DM11 V18I	Ile18	456[c]	42	1.8	1.8

[a] The residue at position 27 in the DCM dehalogenase sequence of strains DM2 and DM4 corresponds to Val18 in that of strain DM11.
[b] K_m obtained from the Michaelis-Menten equation.
[c] $S_{0.5}$ obtained from the Hill equation.

imply allosteric effects, but may arise from certain combinations of individual enzymatic kinetic constants (18). The only instance so far in which sigmoidicity was reported for a GST enzyme was for human class Pi GST, where the introduction of point mutations at C-terminal end of helix 2 led to positive cooperativity with respect to activity and glutathione binding (19).

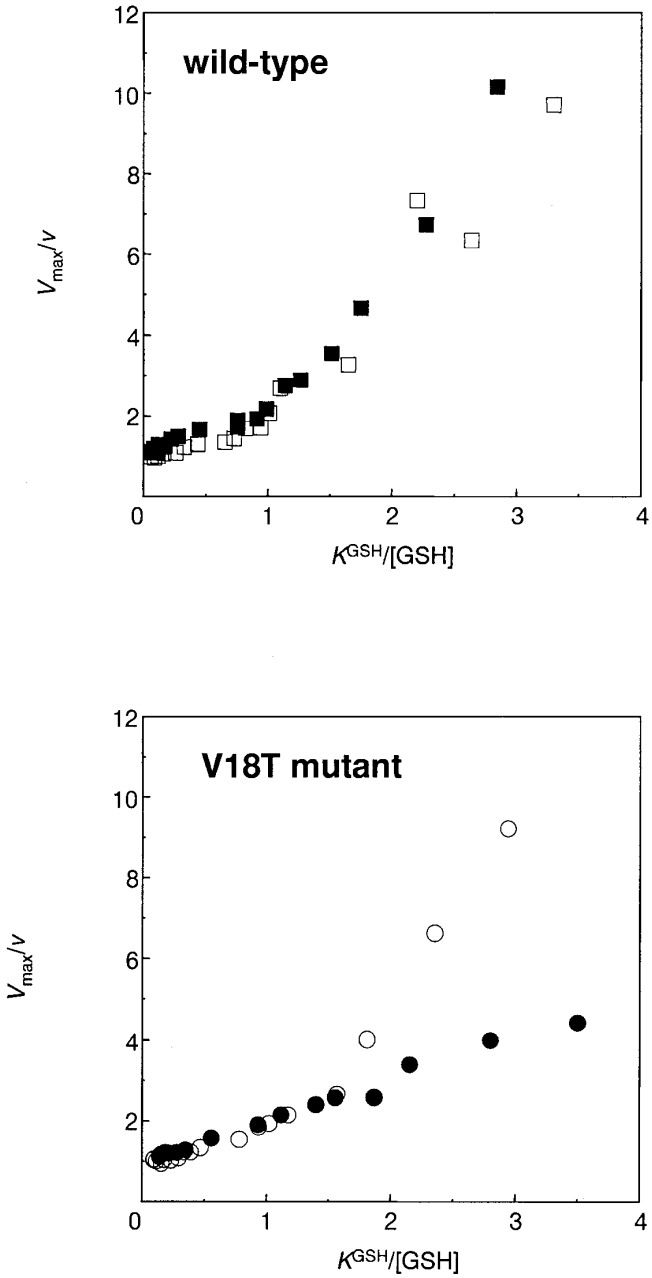

FIG. 2. Double reciprocal plots of the dependence of the enzymatic rate on the concentration of the glutathione cofactor for wild-type dichloromethane dehalogenase from strain DM11 (squares) and for the corresponding V18T mutant protein (circles). Measurements performed at a low, sub-K_m concentration of dichloromethane (20 μM) are shown by filled symbols, and measurements at a saturating concentration of dichloromethane (1 mM) by open symbols, respectively. Units on both axes are expressed as multiples of $1/K_m$ and $1/V_{max}$ to facilitate the comparison between wild-type and mutant proteins.

TABLE 2

Ligand-Dependent Sigmoidicity of Enzymatic Reaction Rates as Expressed by the Hill Coefficient n_H in Protein Variants of Dichloromethane Dehalogenase from *Methylophilus* sp. Strain DM11

	n_H		$S_{0.5}^{GSH}$ (μM)	
DM11 protein variant	1 mM DCM	20 μM DCM	1 mM DCM	20 μM DCM
Wild-type	1.8	1.5	66	94
Val18 → Thr	1.7	1.0	91	112
Val18 → Ala	1.6	1.2	112	89
Val18 → Ile	1.7	1.2	456	254

It will now be of interest to investigate the physiological relevance of differences in glutathione affinity of the key catabolic enzyme in dichloromethane degrading strains, to better understand the selective pressures that have led to the evolution of dichloromethane dehalogenase enzymes within the glutathione *S*-transferase structural scaffold. The intracellular concentration of glutathione in proteobacteria is known to be in the millimolar range, so that a difference in the K_m for glutathione in the 200-400 μM is not expected *a priori* to be critical to the bacterial host. However, the transformation of dichloromethane by dehalogenase enzymes, which are induced to millimolar levels in dichloromethane degrading strains, may lead to severe depletion of the pool of free glutathione. This may be of some importance to the physiology of methylotrophic bacteria during growth on dichloromethane.

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