

Table 1: MALDI-TOF-MS mass fragmentation data of the α -subunit of MCR II from *M. marburgensis*. The drawn sequence of a peptide from the α -subunit contains thioGly447 and methyl-Cys 454. The presence of didehydroaspartate Asp452 (in red) was not included for the calculation of the mass. The precursor mass (2335 Da) is not shown in this table.

Sequence	Molecular mass [$M + H$] ⁺		
	Calcd	Exp	Calcd–Exp
⁴⁴⁴ GFYGYDLQ DD CGASNSLSIR ⁴⁶³	2223.980	2221.764	2.216
⁴⁴⁵ FYGYDLQ DD CGASNSLSIR ⁴⁶³	2166.959	2164.851	2.108
⁴⁴⁶ YGYDLQ DD CGASNSLSIR ⁴⁶³	2019.891	2017.796	2.095
⁴⁴⁷ GYDLQ DD CGASNSLSIR ⁴⁶³	1856.827	1854.611	2.216
⁴⁴⁸ YDLQ DD CGASNSLSIR ⁴⁶³	1783.829	1781.636	2.193
⁴⁴⁹ DLQ DD CGASNSLSIR ⁴⁶³	1620.765	1618.664	2.101
⁴⁵⁰ LQ DD CGASNSLSIR ⁴⁶³	1505.738	1503.688	2.050
⁴⁵¹ Q DD CGASNSLSIR ⁴⁶³	1392.654	1390.560	2.094
⁴⁵² DD CGASNSLSIR ⁴⁶³	1264.596	1262.442	2.154
⁴⁵³ QCGASNSLSIR ⁴⁶³	1149.569	1149.535	0.034
⁴⁵⁴ CGASNSLSIR ⁴⁶³	1021.510	1021.507	0.003
⁴⁵⁵ GASNSLSIR ⁴⁶³	904.485	904.409	0.076

predicted from the nucleotide sequence. MS/MS peptide-sequencing indicated that in this smaller peptide, an amino acid residue at the position of Asp452 had a molecular mass 2 Da smaller than aspartate (Table 1, Figure S4). This finding suggested an oxidation of aspartate to didehydroaspartate. To test this hypothesis, we chemically reduced the 2335 Da peptide in the presence of zinc granule under acidic condition, which yielded a peptide 2 Da larger (Figure S5), likely caused by the reduction of didehydroaspartate to aspartate.

In the crystal structure of MCR II from *M. marburgensis* at 2.15 Å resolution, didehydroaspartate rather than aspartate fits into the electron density at position 452 (Figure S6).

The discovery of a didehydroaspartate residue in MCR II from *M. marburgensis* led us to reconsider the structure of the equivalent aspartate residue in MCR I (Asp450 in the α -subunit) from the same organism. The crystal structures of MCR I are deposited in the PDB.^[8] Although the usual aspartate residue was originally assigned in these structures, the structures at resolutions better than 1.7 Å (the best resolution was 1.16 Å) revealed an approximate 1 Å deviation of the C β atom from the ideal position (the electron density, which has confirmed the C β deviation of the model, was calculated using PDB_REDO^[9]). The electron density of MCR I from *M. marburgensis* matches to the profile of a didehydroaspartate at this position. Peptide-sequencing analysis also indicated that didehydroaspartate exists in the corresponding position of the α -subunit of MCR I (Figure S3 and S7). To obtain the most accurate structural data possible, we crystallized MCR I from *M. marburgensis* and solved the structure at 1.10 Å resolution. In this atomic resolution structure, the Asp450 position was unambiguously modelled with planar didehydroaspartate (Figure 2 and Figure S6). This excellent electron density map revealed a distance of 1.31 Å between Ca and C β which strongly corroborates the presence of the double bond (single-bond distance: 1.54 Å). The double bond between Ca and C β is also clearly indicated by their planarity, which generates a local backbone distortion.

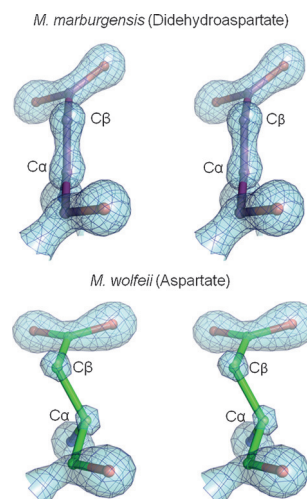


Figure 2. Stereo representation of didehydroaspartate/aspartate at the equivalent position in MCR I from different organisms. The $2F_o - F_c$ electron density clearly indicated the presence of a didehydroaspartate at residue 450 of MCR I from *M. marburgensis* and an aspartate at the equivalent positions of MCR I from *M. wolfeii*. The $2F_o - F_c$ maps are contoured at 4.0σ . For the data of MCR II, see Figure S6.

We analyzed the MCRs from *M. barkeri* (Methanosarcinales) and *M. wolfeii* phylogenetically distantly and closely related to *M. marburgensis*, respectively. MCR from *M. barkeri* has been already structurally characterized.^[4] A re-inspection of the electron density revealed a better fit for a model with didehydroaspartate (Figure S8), although the original PDB file contains an aspartate at this position.^[4] Peptide-sequencing analyses confirmed a didehydroaspartate in MCR from *M. barkeri* (Figures S3,S9). These data suggested that didehydroaspartate is widespread in methanogenic archaea.

Unexpectedly, peptide analysis revealed that both MCR isoenzymes from *M. wolfeii* have the usual aspartate at the equivalent position and not didehydroaspartate even though the MCRs are closely related to the enzymes from *M. marburgensis*, with 99 % (MCR I) and 96 % (MCR II) sequence identity (Figure S3,S10,S11). To confirm this result, we crystallized both MCR isoenzymes from *M. wolfeii* and determined their structures (MCR I, 1.4 Å resolution; and MCR II, 1.8 Å resolution); both contained the canonical aspartate rather than didehydroaspartate (Figure 2 and Figure S6). Moreover, the modification was explored under different culture (batch and fermenter) and growth phase conditions (mid-exponential, late-exponential, and stationary phases); MALDI-TOF-MS peptide analysis detected only a canonical aspartate at the expected modified site.

Didehydro amino acids found as didehydroaspartate of MCR are amino acids with a new type of modification in proteins. Didehydroaspartate in MCR is encoded by the standard Asp codon (for sequence, see Figure S1,S12) implicating that the oxidation of the aspartate residue is post-translationally performed. The only known natural compound containing didehydroaspartate is a cyclic hexapeptide, phomopsis A; this mycotoxin is produced by the fungus *Phomopsis leptostromiformis*^[10] and is responsible for lupinosis

disease in animals.^[11] The biosynthetic pathway of phomopsis A including the formation of the didehydroaspartate moiety, has not yet been elucidated. Since the didehydroaspartate residue could be only detected by high-resolution X-ray crystallography or peptide analysis using accurate mass spectrometry, its presence in proteins may have been overlooked. Perhaps other proteins also contain didehydroaspartate.

Post-translational modifications of amino acids in proteins can occur auto-catalytically^[12] or enzymatically.^[5a,d,6,13] No enzyme, characterized so far, is able to catalyze the formation of didehydro amino acid in proteins. The well-characterized aerobic and anaerobic aspartate oxidases, which oxidize free aspartate to iminoaspartate^[14] are presumably not suitable for the post-translational reaction. A comparative genomics analysis indicated that eight oxidoreductase genes are present in *M. marburgensis* but absent in *M. wolfeii*. One of these genes was annotated as a pyrroline-5-carboxylate reductase homologue; this enzyme might catalyze a dehydrogenation reaction similar to that of didehydroaspartate formation. A pyrroline-5-carboxylate reductase homologue is also found in *M. barkeri* (Mbar_A0480).

Accurate functional studies on pure MCR are extremely difficult due to its complex activation and the instability of the active Ni^I state of coenzyme F₄₃₀. The growth rate of methanogens can be an indicator of the in vivo activity of MCR because MCR catalyzes the rate-limiting reaction in methane formation from H₂ and CO₂. Our experiments demonstrated that the growth rate of *M. marburgensis* is about 1.6-fold faster than that of *M. wolfeii* (in the exponential phase) under the same culture conditions and the content of MCR was almost the same (Figure S13). This finding indicated that the in vivo activity of MCRs with and without didehydroaspartate might only slightly differ.

The structural data show that in both MCRs with/without didehydroaspartate, the carboxy group of the (didehydro)aspartate residue interacts with His484 and Trp427 (numbering as in *M. marburgensis* MCR I) which stabilizes the loop regions contacting coenzyme B and coenzyme M (Figure 3).

Nevertheless, the introduction of a double bond into an aspartate has substantial chemical and structural consequences which affect the active-site and potentially also the catalytic activity of MCR. The C α =C β double bond of didehydroaspartate narrows the conformational space of the side chain, and therefore fixes the carboxylate group in a single direction and reduces its mobility. In addition, the carboxy group conjugated with the C α =C β double bond lowers the pK_a value (<http://www.chem.wisc.edu/areas/organic/index-chem.htm>).^[15] Thus, the didehydroaspartate modification appears to fine-tune the strength of the ionic interactions, side chain positions, local backbone conformations (Figure S14) and polypeptide motions to optimize substrate-binding and catalysis. Since no crystal structure of MCR in the Ni^I active state has been solved so far, the advantage of didehydroaspartate versus aspartate for the catalytic process remains unknown.

The introduction of post-translational modifications into amino acid residues is energetically expensive. Why did nature then employ this strategy for MCR? One reason might

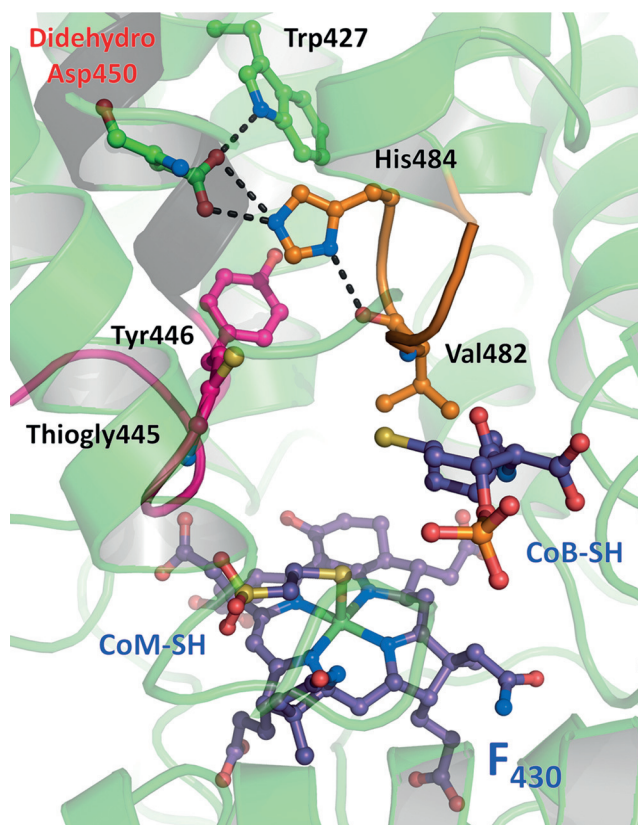


Figure 3. The loop region with the didehydroaspartate residue in MCR I from *M. marburgensis*. Didehydro-Asp450 is hydrogen-bonded with His484^a and Trp427^a and appears therefore to be a key element to fix the coenzyme B and coenzyme M binding loops (orange and magenta, respectively). A fine-tuning of geometry and polypeptide motions of the active site might promote the higher catalytic activity.

be that the active site structure cannot be further optimized by ordinary substitutions of amino acid residues. Only smaller structural changes realized by post-translational modification might be advantageous to further fine-tune the complex reaction cycle of reductive sulfur–carbon bond cleavage and anaerobic methane oxidation. Because the MCR-catalyzed reaction is the rate-limiting step of methane formation from H₂ and CO₂,^[16] the enhancement of the enzymatic power of MCR would directly increase the turnover or would allow a reduction of the enormous amounts of MCR molecules in the cell. Moreover, the functional significance of the post-translational modification might become only visible under special cellular and environmental conditions.

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