Protein Structures

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Didehydroaspartate Modification in Methyl-Coenzyme M Reductase Catalyzing Methane Formation

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Abstract: *All methanogenic and methanotrophic archaea known to date contain methyl-coenzyme M reductase (MCR) that catalyzes the reversible reduction of methyl-coenzyme M to methane. This enzyme contains the nickel porphinoid F_{430} as a prosthetic group and, highly conserved, a thioglycine and four methylated amino acid residues near the active site. We describe herein the presence of a novel post-translationally modified amino acid, didehydroaspartate, adjacent to the thioglycine as revealed by mass spectrometry and high-resolution X-ray crystallography. Upon chemical reduction, the didehydroaspartate residue was converted into aspartate. Didehydroaspartate was found in MCR I and II from Methanothermobacter marburgensis and in MCR of phylogenetically distantly related Methanosarcina barkeri but not in MCR I and II of Methanothermobacter wolfeii, which indicates that didehydroaspartate is dispensable but might have a role in fine-tuning the active site to increase the catalytic efficiency.*

Methyl-coenzyme M reductase (MCR) commonly catalyzes the methane-forming step in all methanogenic archaea[1] and the oxidation of methane, in anaerobic methanotrophic archaea.[2] MCR is a dimer of heterotrimers composed of α-, β-, and γ-subunits. The active site deeply embedded in the protein interior is positioned in front of the prosthetic group F_{430}. This redox-active nickel porphinoid is directly involved in the cleavage of the methyl–sulfur bond of methyl-coenzyme M to form methane and the coenzyme M/coenzyme B heterodisulfide (Figure 1). Many hydrogenotrophic methanogenic archaea synthesize two MCR isoenzymes: MCR I and MCR II. Their primary structures are 60–70% identical (Supporting Information, Figure S1, Table S1).[3] Crystal structures of MCRs from *Methanothermobacter marburgensis* (MCR I),[1c] *Methanosarcina barkeri*,[4] and *Methanopyrus kandleri*[4] have been solved, but to date no crystal structure of MCR II has been reported.

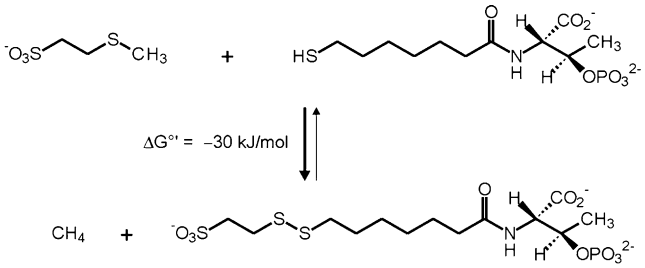

Figure 1. Reaction catalyzed by methyl-coenzyme M reductase.

Nature expands the functions of proteins by post-translational amino acid modifications[5] such as, methylation, acetylation, carboxylation, formylation, and carbamylation. The α-subunit of MCR contains five post-translationally modified amino acid residues near the active site: 1-*N*-methylhistidine, *S*-methylcysteine, 5-C-(*S*)-methylarginine, 2-C-(*S*)-methylglutamine, and thiopeptide-bound thioglycine.[1c] Stable-isotope-labelling experiments indicated that the methyl groups of the methylated residues are provided by methionine, most probably via *S*-adenosylmethionine.[6] Recent crystal structure analysis of MCR from anaerobic methanotrophic archaea (ANME-1) suggested in addition the presence of C^7-hydroxy tryptophan and *S*-oxymethionine.[7] The physiological functions of the modified amino acids are not known.

In this study, we performed peptide-sequencing and crystal structure analyses of MCRs from *Methanothermobacter marburgensis*, *Methanothermobacter wolfeii* and *Methanosarcina barkeri.* Our investigations revealed the presence of a novel post-translationally modified amino acid, didehydroaspartate, in MCRs of *M. marburgensis* and phylogenetically distant *M. barkeri*, but not in *M. wolfeii.*

MCR II from *M. marburgensis* was purified and digested with trypsin, and peptide fragments were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS). Peptide fragments containing methylglutamine, methylhistidine, or methylarginine were detected. According to the primary structure, methylcysteine and thioglycine should be part of one peptide fragment; however, such a peptide (from Leu 443 to Arg 463; theoretical mass 2337 Da) was not detected, even though the five modified amino acid residues found in MCR I were observed in the crystal structure of MCR II (Figure S2). In further peptide sequencing experiments, the corresponding amino acid sequence was found in a 2335 Da fragment (Table 1 and Figure S3); this peptide was 2 Da smaller than the peptide

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Supporting information (technical details, procedures, sequences and structures are provided in the Supplementary Information. X-ray analysis statistics and PDB codes are shown in Table S2) for this article can be found under: http://dx.doi.org/10.1002/anie.201603882.

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-Cys454. 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}GFY**G**YDLQ**D**Q**C**GASNSLSIR^{463}	2223.980	2221.764	2.216
^{445}FY**G**YDLQ**D**Q**C**GASNSLSIR^{463}	2166.959	2164.851	2.108
^{446}Y**G**YDLQ**D**Q**C**GASNSLSIR^{463}	2019.891	2017.796	2.095
^{447}**G**YDLQ**D**Q**C**GASNSLSIR^{463}	1856.827	1854.611	2.216
^{448}YDLQ**D**Q**C**GASNSLSIR^{463}	1783.829	1781.636	2.193
^{449}DLQ**D**Q**C**GASNSLSIR^{463}	1620.765	1618.664	2.101
^{450}LQ**D**Q**C**GASNSLSIR^{463}	1505.738	1503.688	2.050
^{451}Q**D**Q**C**GASNSLSIR^{463}	1392.654	1390.560	2.094
^{452}**D**Q**C**GASNSLSIR^{463}	1264.596	1262.442	2.154
^{453}Q**C**GASNSLSIR^{463}	1149.569	1149.535	0.034
^{454}**C**GASNSLSIR^{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 Cβ atom from the ideal position (the electron density, which has confirmed the Cβ 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 Cα and Cβ which strongly corroborates the presence of the double bond (single-bond distance: 1.54 Å). The double bond between Cα and Cβ is also clearly indicated by their planarity, which generates a local backbone distortion.

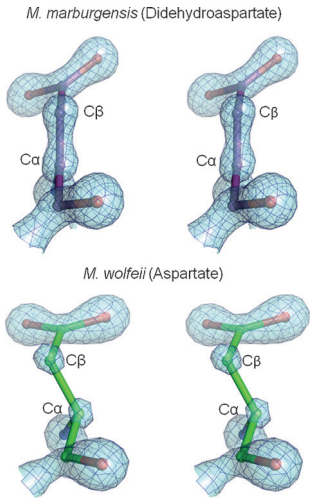

Figure 2. Stereo representation of didehydroaspartate/aspartate at the equivalent position in MCR I from different organisms. The $2F_o-F_c$ electron density clearly indicated the presence of a didehydroaspartate at residue 450 of MCR I from *M. marburgensis* and an aspartate at the equivalent positions 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, phomopsin A; this mycotoxin is produced by the fungus *Phomopsis leptostromiformis*[10] and is responsible for lupinosis

disease in animals.[11] The biosynthetic pathway of phomopsin 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 F_{430}. 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 H_2 and CO_2. 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 His 484 and Trp 427 (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 Cα=Cβ 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 Cα=Cβ 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

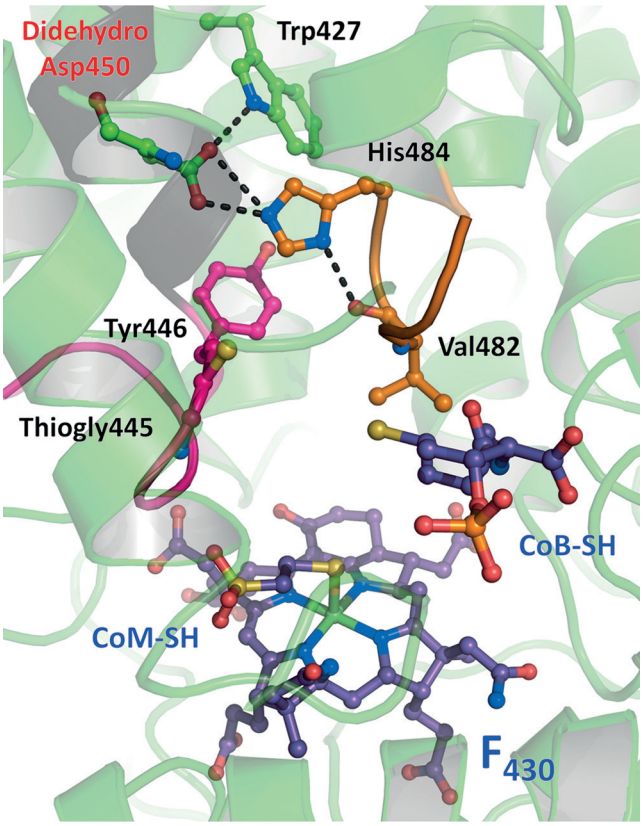

Figure 3. The loop region with the didehydroaspartate residue in MCR I from *M. marburgensis*. Didehydro-Asp 450 is hydrogen-bonded with His 484$^{\alpha}$ and Trp 427$^{\alpha}$ and appears therefore to be a key element to fix the coenzyme B and coenzyme M binding loops (orange and magenta, respectively). 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 H_2 and CO_2,[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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