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Supporting Information

A Cobalamin-Dependent Radical SAM Enzyme Catalyzes the Unique C_α-Methylation of Glutamine in Methyl-Coenzyme M Reductase

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Experimental Procedures

Materials

Chemicals, media ingredients and other reagents were purchased from Carl Roth GmbH & Co. KG or Sigma-Aldrich/Merck unless stated otherwise. Enzymes for molecular biology techniques and molecular weight markers for agarose gel electrophoresis and SDS-polyacrylamide gel electrophoresis were purchased from New England BioLabs GmbH. Oligonucleotides were obtained from Eurofins Genomics. Wizard SV Gel and PCR Clean-up System and Wizard Plus SV Minipreps DNA Purification System were purchased from Promega. Peptide substrates were custom-synthesized by TAG Copenhagen A/S. For tryptic digestion of peptides, Trypsin (Sigma) from porcine pancreas Type IX-S was used. Chemicals used for mass spectrometry were in LC-MS grade. DL-2-Methylglutamic acid was purchased from ABCR. LC-MS grade formic acid and acetonitrile were purchased from Fisher.

Methods

Construction of the *SAMN04488571_10412* expression plasmid
Genomic DNA of *Methanoculleus thermophilus* DSM2373 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). *E. coli* DH10B was used as the host for cloning. The *SAMN04488571_10412* gene was amplified by PCR using the Phusion Polymerase (generated in-house) with forward primer
5'- CGGCATATGGATATCACGATCTTCTCCC -3' and reverse primer
5'- GACCTCGAGTCTTTGGGCGTAGCGGAGG -3'. PCR fragments were digested with *Nde*I and *Xho*I (underlined) and purified using the Wizard SV Gel and PCR Clean-up System. Vector pET-21a(+) (Novagen) was correspondingly digested and purified by agarose gel electrophoresis and gel extraction. Digested PCR fragments were ligated into the linearized vector pET-21a(+) using T4 DNA ligase. The resulting construct (pET21a-QCMT) codes for QCMT carrying a C-terminal His_6-tag. The correct sequence of the construct was verified by sequencing (Eurofins Genomics).

Heterologous production of recombinant QCMT
E. coli BL21(DE3) star pLysS was transformed with plasmids pET21a-QCMT and pBAD42-BtuCEDFB[1] encoding the *btuCEDFB* operon from *E. coli*. Bacteria containing both plasmids were selected on LB agar plates with 34 µg ml^{-1} chloramphenicol, 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} spectinomycin. Recombinant protein production was done in 2 l flasks containing 1 l LB medium and the previously mentioned antibiotics. After inoculation to an A_{600} ~ 0.03 with an overnight culture, cultures were incubated at 37 °C with shaking at 150 rpm until they reached an A_{600} ~ 0.3. At this point, expression of the cobalamin transporter genes was induced with 0.2 % arabinose (w/v) and 1.3 µM OHCbl was added. After reaching an A_{600} ~ 0.5, cultures were supplemented with 1 mM ammonium ferric citrate and 1 mM cysteine, and production of QCMT was induced with 0.1 mM IPTG. Cultures were then incubated overnight at 20 °C and 130 rpm. Cells were collected by centrifugation at 4 °C at 4816 *g* for 20-30 min and stored in an anaerobic bottle at -20 °C.

Purification of recombinant His_6-tagged QCMT
All purification steps and further handling of the purified protein were done under anaerobic conditions in an anaerobic chamber containing 95% N_2 and 5% H_2 (Coy Laboratory Products Inc.). Cells were thawed overnight at 7 °C in anaerobic lysis buffer B (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 % glycerol, 20 mM imidazole) in a 1:2.5 ratio (w/v). Lysozyme (~ 0.5 mg/ml) and 100 µl DNAse (2 mg/ml stock solution) were added and cells were stirred for 30 min at 7 °C. The suspended cells were disrupted with a FastPrep-

24™ bead beating lysis system (MP Biomedical) by shaking three times for 40 s at 5.5 m/s. In between, the cell suspension was cooled on ice for about 5 min. For sedimentation of the glass beads, the crude extract was centrifuged at 2790 *g* for 5 min at 4 °C. Afterwards, the soluble protein fraction was obtained by centrifugation at 125,082 *g* for 1 h at 10 °C.
His_6-tagged QCMT was purified by immobilized metal affinity chromatography (IMAC) using an Äkta purifier 10 FPLC system (GE Healthcare). The soluble protein fraction was loaded on a 4 ml His60 Ni Superflow Resin (Takara Bio Inc.) column equilibrated with lysis buffer B at a flow rate of 1 ml/min. Unbound proteins were washed off the column with buffer B at a flow rate of 1 ml/min until the UV signal reached a stable base line. Proteins bound to the column were eluted with 50 % elution buffer C (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 500 mM imidazole). Elution fractions were analyzed by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the QCMT-containing fractions were pooled. Buffer exchange against storage buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 % glycerol) was done by using a PD-10 column (Cytiva Life Sciences) according to the manufacturer's instructions. Protein solutions were concentrated using Amicon centrifugal filter devices (Millipore) with 30 kDa cutoff.

Determination of protein concentration
Protein concentration was determined with Bradford reagent (Bio-Rad Laboratories, Inc.) using BSA as a standard according to the manufacturer's protocol.

Chemical reconstitution of cofactors
For reconstitution of the cobalamin cofactor, as-isolated QCMT (after IMAC) was incubated with equimolar amounts of either methylcobalamin or hydroxocobalamin for 1 to 2 h at room temperature. Unbound cobalamin was separated from the protein by using a PD-10 column.
Reconstitution of the iron-sulfur cluster was performed before and after size exclusion chromatography according to Flühe *et al*.[2] with the following modifications. Storage buffer A was used for the preparation of the required solutions. All incubation steps were done at room temperature. The protein solution was incubated with 4 - 5 equiv. of ammonium iron citrate for 15 - 20 min and with 4 - 5 equiv. of lithium sulfide for 1 - 1.5 h. After reconstitution, the protein solution was centrifuged in order to remove any precipitated material, and excess of ammonium iron citrate and lithium sulfide was removed using a PD-10 column or by size exclusion chromatography.

Size exclusion chromatography
For further purification, the reconstituted QCMT was centrifuged for 10 min (16,100 *g*) and loaded on a HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare) equilibrated with storage buffer A at a flow rate of 1 ml/min. Elution fractions were analyzed by SDS-PAGE and the fractions containing monomeric QCMT were pooled.
Analytic size exclusion chromatography was performed on Superdex 200 Increase 5/150 GL (Cytiva) equilibrated with storage buffer A at a flow rate of 0.25 ml/min. A gel filtration standard (Biorad) was employed to generate a standard curve.

Determination of iron and sulfide contents
The iron and sulfide contents of purified and reconstituted QCMT was determined as previously described.[3]

UV/Visible absorption spectroscopy
UV/Vis absorption spectra were recorded with a V-650 spectrophotometer (Jasco) in a quartz glass cuvette placed in an anaerobic chamber with a sample volume of 100 µl sample. Spectra were normalized by setting the absorption at 750 nm to zero.
The cobalamin content of as-isolated QCMT and as-purified QCMT was analyzed by mixing 50 µM QCMT with 150 µM H_2SO_4 and boiling for 10 min at 70 °C. The denatured enzyme was separated by centrifugation (16,100 *g*, 10 min) and the supernatant was analyzed by UV/Vis spectroscopy.
UV/Vis absorption spectra of titanium(III) citrate reduced QCMT were recorded after incubation of 20 µM as-purified QCMT with 80 µM titanium(III) citrate for 3 h. Then, 40 µM SAM were added to reduced QCMT and spectra were measured after incubation for an additional 30 min. Next, 20 µM peptide substrate (QS-28) were added and spectra were measured instantly. After each step, samples were taken and quenched with formic acid for HPLC analysis of SAH and DOA formation. Control reactions contained reduced QCMT with peptide substrate but without SAM, and reduced QCMT incubated with SAM and a peptide substrate with a different amino acid sequence (HAALVSMGEMLPARRARGPNEPGG). In addition, after the final step, samples were taken for LC-MS analysis of the peptide substrate.
For the reduction of QCMT with sodium dithionite (DT), as-purified QCMT (9 µM) was incubated with equimolar amounts of DT for 1.5 h and spectra were measured. Then, 18 µM SAM were added and spectra were measured after incubation for 15 min.

***In vitro* enzyme activity assays**
For the design of the peptide substrate the available amino acid sequence fragments of *Methanoculleus thermophilus* McrA subunit from Uniprot (https://www.uniprot.org/) were compared by multiple sequence alignments (https://www.ebi.ac.uk/Tools/msa/clustalo/). Hence, two peptides were designated as potential substrates: the peptide QS-28 consisting of the 28 amino acids QFPTMMEDHFGGS**Q**RAGVIAAASGLSTS and the peptide PS-24 comprising the 24 amino acids PTMMEDHFGGS**Q**RAGVIAAASGLS. These substrates were obtained by custom-synthesis (TAG Copenhagen A/S).
In vitro enzyme activity assays were performed under anaerobic conditions. Reaction mixtures were set up with as-purified QCMT reconstituted with MeCbl in storage buffer A and incubated at 37 °C for 4 h unless stated otherwise. The reaction was stopped by adding formic acid [final concentration of 5 % (v/v)]. The initial *in vitro* reaction mixture for HPLC and LC-MS analysis contained 25 µM QCMT, 200 µM SAM, 50 µM peptide substrate (QS-28), and 1 mM sodium dithionite in a total volume of 300 µl. The final standard activity assay for HPLC analysis of SAM cleavage products contained 1 µM QCMT, 200 µM SAM, 50 µM peptide substrate (either QS-28 or PS-24), 1 mM titanium(III) citrate in a total volume of 100 µl. For analysis of the time-dependent formation of DOA and SAH a higher total volume was employed, and samples of 100 µl were taken at the respective time points and analyzed by HPLC. For quantification of SAH and DOA formation, standard solutions containing 5, 10, 15, 20, 25, 50, 75, and 100 µM of either SAH or DOA were prepared and analyzed by HPLC.
For LC-MS analysis of peptides, assays were performed with 2 µM as-purified QCMT (OHCbl-Rc), 200 µM SAM, 50 µM peptide substrate (either QS-28 or PS-24), 1 mM titanium(III) citrate in a total volume of 300 µl and incubated for 3.5 h. Assays with deuterium-labeled methionine contained 2 µM as-purified QCMT (OHCbl-Rc), 11 µM *Ec*MAT,[4] 2 mM L-methionine (methyl-d^3, Eurisotop), 2 mM

ATP, 50 µM peptide substrate (either QS-28 or PS-24), 1 mM titanium(III) citrate, 20 mM $MgCl_2$ and 50 mM KCl and were incubated for 4 h.

Assay mixtures for determination of the pH optimum contained 1 µM QCMT, 200 µM SAM, 50 µM peptide substrate (PS-24), 1 mM titanium(III) citrate, 300 mM NaCl, 10 % (v/v) glycerol and 50 mM of one of the following buffers in a total volume of 200 µl: MES, pH 6.5; HEPES, pH 7.0; Tris-HCl, pH 7.5/ 8.0/ 8.5/ 9.0; CAPSO, pH 9.5/ 10.0; and CAPS, pH 10.5/ 11.0. Reaction mixtures were pre-incubated at 37 °C and started with the addition of SAM after 10 – 15 min. Samples of 100 µl were quenched after 20 min and 40 min, respectively, and analyzed by HPLC. The initial velocity v_0 was calculated as $v_0 = \frac{\Delta\, cDOA}{\Delta\, t}$ in µM DOA/min/µM QCMT.

The assay for LC-MS analysis of enzyme-bound cobalamin contained 10 µM as-purified QCMT, 11 µM *Ec*MAT, 1 µM *Ec*MTAN, 2 mM L-methionine or L-d^3-methionine, 2 mM ATP, 1 mM titanium(III) citrate, 50 µM peptide substrate, 20 mM $MgCl_2$ and 50 mM KCl in a volume of 120 µL and were incubated for 4 h.

HPLC analysis of SAM cleavage products

Before HPLC analysis, the formic acid quenched samples were centrifuged for 10 min at 21,130 *g*. HPLC analysis was carried out either on a Jasco LC-2000Plus or LC-4500 series system with either a Hypercarb column (Thermo fisher, 100 x 2.1 mm, particle size 5 µm) or a Reprosil-Pur ODS-3 column (Dr. Maisch Gmbh, 125 mm x 3 mm, particle size 3 µm). The Hypercarb column was equilibrated with solvent A (double distilled water + 0.1 % (v/v) trifluoroacetic acid). Separation of the samples was done applying the following gradient at a flow rate of 0.2 ml/min: 0 - 23 min linear gradient up to 30 % A/ 70 % solvent B (acetonitrile + 0.1 % (v/v) trifluoroacetic acid), 23 - 25 min linear gradient up to 0 % A/ 100 % B, 25 - 30 min 0 % A/ 100 % B, 30 - 35 min linear gradient up to 100 % A/ 0 % B, 35 - 45 (LC-2000Plus) or 35 - 50 min (LC-4500) 100 % A. Detection was performed at 260 nm.

When using the Reprosil-Pur ODS-3 column connected to the Jasco LC-4500 series system, the column was equilibrated with 97 % solvent A (1 % (w/v) ammonium acetate, pH 5.1) and 3 % solvent B (acetonitrile) at a flow rate of 0.2 ml/min. The following gradient was used for separation: 0 – 5 min initial conditions, 5 – 10 min linear gradient up to 35 % B, 10 – 20 min 35 % B, 20 – 25 min returning to 97 % A/ 3 % B and 25 – 45 min 97 % A/ 3 % B.

LC-MS and MS/MS analysis of peptide substrates

The formic acid quenched samples were centrifuged for 10 min at 21,130 *g* and filtered through a syringe filter (PTFE, Ø 4 mm, Pore 0.2 µm, ISERA) by centrifugation (2 - 10 min, 4,500 *g*). For peptide LC-MS measurements, the peptides were separated from other assay components with an ISAspher 300 Å – 5 µm C4 150 x 2,0 mm HPLC column on an HPLC system (Agilent 1100, USA) connected to an ABSciex Qtrap 4500 (AB Sciex, Darmstadt, Germany) hybrid ESI mass spectrometer. Mobile phase A consisted of water containing 0.1 % formic acid, and mobile phase B consisted of acetonitrile containing 0.1 % formic acid. Separation was achieved with a 40 min method using a flow rate of 0.2 ml/min. Used gradient for separation was as follows, initial: 10 % B up to 1 min, linear gradient until 8 min increasing B to 70 %, up to 15 min linear gradient increasing B to 90 %, followed by washing with 90 % B until 23 min and returning to initial conditions and re-equilibration. Mass spectrometer measurements were performed in positive ionization mode with the following parameters: Curtain gas 30 psi, ion Spray voltage 4500 V, temperature: 200 °C, entrance potential 10 V, declustering potential 100 V, nebulizer gas 40 psi, heater gas 50 psi. For measurements of peptide methylation Enhanced MS (EMS) LIT-Scan mode was performed with the following additional parameters: collisionally activated dissociation (CAD) high, collision energy 5 V and enabled dynamic fill-time. MS/MS peptide fragmentations were performed using enhanced product ion (EPI) LIT-scan mode at 27 V collision energy. For LC-MS peptide measurements 5 µl of assay mixture containing 50 µM peptide was injected. For MS/MS fragmentation after trypsinolysis, 15 µl were injected. LC-MS spectra were deconvoluted and visualized with UNiDec Version 4.2.2.[5] MS/MS spectra were visualized and edited with IPSA[6] and Inkscape (retrieved from https://inkscape.org).

Peptide substrate isolation and trypsinolysis for MS/MS analysis

The peptide was isolated from the assay mixture via HPLC (Agilent 1260 Infinity II, USA) with the same method used for LC-MS peptide measurements. The isolated peptide was freeze-dried and dissolved in 100 mM Tris-buffer pH 7.8, matching the volume injected into the HPLC before. Trypsin was added in a ratio of 1/50 (w/w) and the sample was incubated at 37 °C overnight. Desalting and separation of the digested peptide before MS/MS fragmentation was achieved by described LC method.

LC-MS analysis of amino acids from peptide hydrolysis

Before amino acid analysis via LC-MS, enzyme was removed with vivaspin 500 centrifugal concentrator (10,000 MWCO). LC-MS analysis was conducted after acid hydrolysis (6 N HCl, 120 °C, 24 h) of methylated QS-28 peptide (QCMT added) and not methylated QS-28 peptide (control lacking enzyme). Glutamine species were not detected due to the formation of glutamate under strongly acid conditions. For LC-MS measurements, the amino acids were separated with a Syngergi 4 µm Fusion-P 50 X 2 HPLC column on an HPLC system (Exion LC, Germany) connected to an ABSciex Qtrap 5500 (AB Sciex, Darmstadt, Germany) ESI mass spectrometer. Mobile phase A consisted of water containing 0.1 % formic acid, and mobile phase B consisted of acetonitrile. The duration of the method was 5 min and an isocratic of 100 % mobile Phase A was used.

Mass spectrometer measurements were performed in positive ionization mode with the following parameters: Curtain gas 25 psi, ion Spray voltage 4500 V, temperature: 450 °C, entrance potential: 10 V, nebulizer gas 40 psi, heater gas 50 psi. Following MRM transitions were optimized with standards:

ID	Q1	Q3	Dwell Time (msec)	DP (V)	CE (V)	CXP (V)
glutamate	148.03	130.07	100	50	14	10
glutamate	148.03	84.05	100	50	22	10
glutamate	148.03	102.05	100	50	16	10
2-methylglutamate	162.03	98.05	100	50	24	10
2-methylglutamate	162.03	116.08	100	50	19	10
2-methylglutamate	162.03	144.09	100	50	15	10

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LC-MS analysis of enzyme-bound MeCbl

Samples were centrifuged for 15 min at 12,100 *g* and filtered through a syringe filter (PTFE, Ø 4 mm, Pore 0.2 µm, ISERA) for 10 min at 3,100 *g*. The samples were stored on ice until measurement. Measurement of enzyme-bound MeCbl was possible due to denaturing conditions during the separation on the HPLC column. Separation was achieved by running the described HPLC method from LC-MS peptide measurements. 20 µl of assay mixture containing 10 µM enzyme were injected. For mass spectrometric detection of MeCbl, precursor-Ion scan mode was performed. The Precursor was set to 665.3 Da, corresponding to the common daughter fragment of cobalamin species.

EPR spectroscopy

For EPR analysis of the iron-sulfur cluster, 175 - 200 µM as-purified QCMT were incubated (1) with 5 mM DT, (2) with 5 mM DT plus 1 mM SAM and (3) with 5 mM DT plus 1 mM SAM plus 300 µM peptide substrate (PS-24) at room temperature for 10 - 20 min in an anaerobic chamber. Samples were transferred to an EPR tube and frozen in liquid nitrogen. Samples were stored in liquid nitrogen until measurement. Samples for the analysis of the cobalamin cofactor contained (1) 175 – 200 µM as-purified QCMT, (2) incubated with 1 mM SAM, (3) incubated with 300 µM peptide substrate (PS-24) and (4) incubated with 1 mM SAM plus 300 µM peptide substrate (PS-24).

Each EPR sample was likewise analyzed by UV/Vis absorption spectroscopy in a 1:20 dilution.

EPR measurements were conducted with an EMX 6/1 ESR spectrometer (Bruker) operating at X-band. The sample temperature was controlled with an ESR-900 helium flow cryostat (Oxford Instruments). The magnetic field was calibrated using a strong pitch standard. EPR conditions were: microwave frequency, 9.44 GHz; modulation amplitude, 0.63 mT; modulation frequency, 100 kHz; time constant 0.164 s; scan rate, 17.9 mT/min. The individual experimental conditions are provided in the figure legends.

Results and Discussion

Figure S1

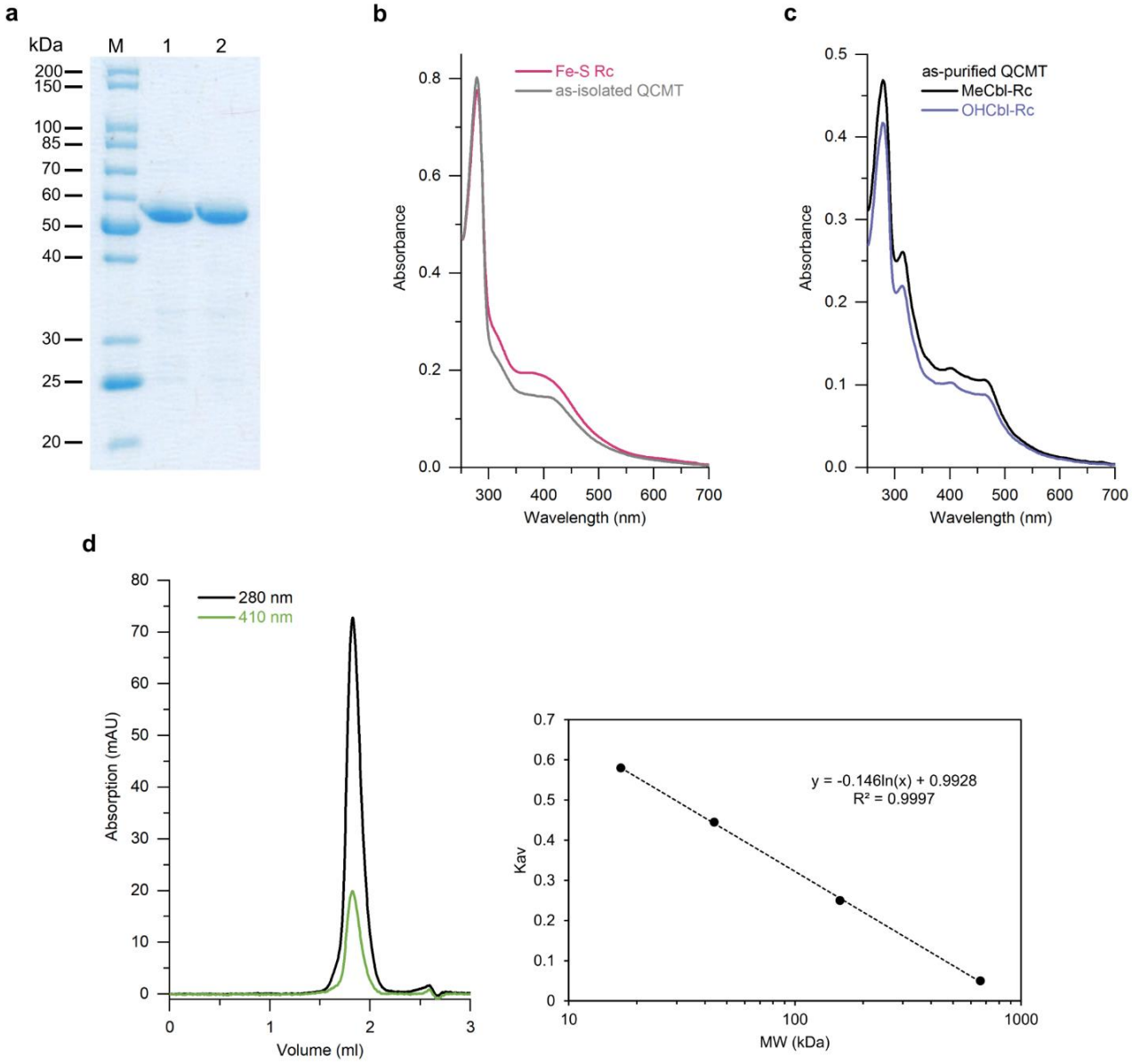

Figure S1. QCMT purification and cofactor reconstitution. (a) SDS-PAGE analysis of QCMT after IMAC and SEC. Lane 1: pooled QCMT-containing fractions after IMAC, lane 2: pooled QCMT-containing fractions after SEC, lane M: molecular weight standard. (b) UV/Vis absorption spectra of as-isolated QCMT and Fe-S reconstituted QCMT (c) UV/Vis absorption spectra of as-purified QCMT reconstituted with either MeCbl or OHCbl (d) Determination of the oligomeric state of QCMT. Chromatogram of analytic SEC (left) and standard curve for analytic SEC (right). Standards were: thyroglobulin, γ-bovine globulin, chicken ovalbumin and equine myoglobin.

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Figure S2

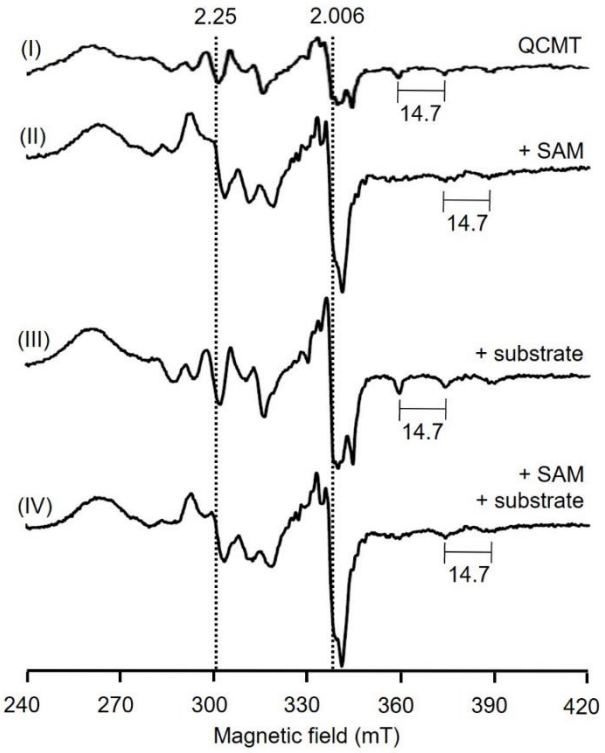

Figure S2. Characterization of QCMT-bound cobalamin by EPR spectroscopy. EPR spectra at 65 K of (I) as-purified QCMT, (II) as-purified QCMT after addition of SAM, (III) as-purified QCMT after addition of peptide substrate and (IV) as-purified QCMT after addition of peptide substrate and SAM. The hyperfine splitting is given in mT and the position of signals discussed in the text is indicated. Other EPR conditions: microwave power, 20 mW (non-saturating), nine scans.

Figure S3

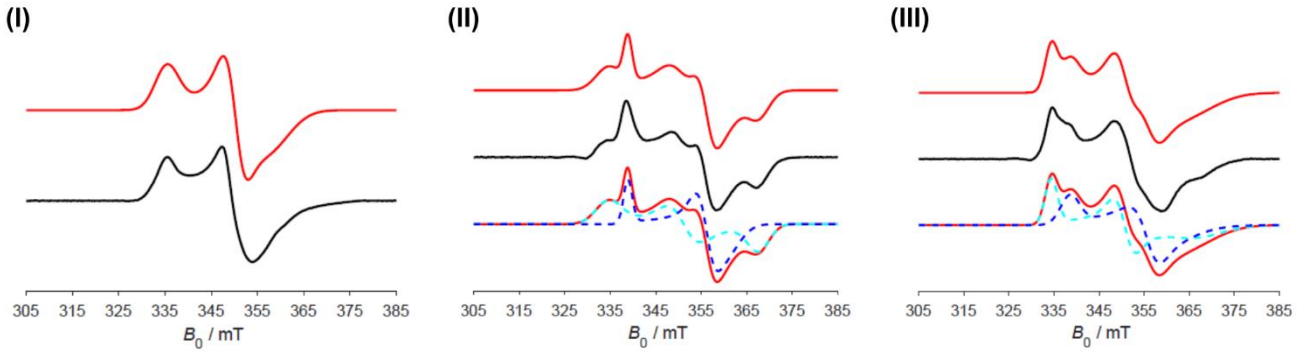

Figure S3. EPR spectra of the double-baseline corrected experimental spectra (black) and their simulation (red) of reduced QCMT (I), reduced QCMT plus SAM (II) and the reduced QCMT plus SAM and substrate (III). In (II) and (III) the contribution of the individual species (system (1) in cyan and system (2) dark blue) to the simulated spectrum (red) is shown. The spectra were simulated by using the EasySpin algorithm "pepper".[7] The simulations were performed by assuming a rhombic *g*-tensor together with a collinear Gaussian distributed *g*-strain model. For (II) and (III) two independent systems (1) and (2) were used. The resulting parameters were as follows:

(I) $g_{x,y,z}$ = 2.017, 1.930 and 1.888; $g\text{-strain}_{x,y,z}$ = 0.036, 0.024 and 0.055

(II) $g(1)_{x,y,z}$ = 2.022, 1.927 and 1.841; $g\text{-strain}(1)_{x,y,z}$ = 0.040, 0.037 and 0.028
$g(2)_{x,y,z}$ = 1.996, 1.898 and 1.875; $g\text{-strain}(2)_{x,y,z}$ = 0.013, 0.021 and 0.038

(III) $g(1)_{x,y,z}$ = 2.022, 1.927 and 1.841; $g\text{-strain}(1)_{x,y,z}$ = 0.019, 0.024 and 0.068
$g(2)_{x,y,z}$ = 1.996, 1.898 and 1.875; $g\text{-strain}(2)_{x,y,z}$ = 0.028, 0.027 and 0.084

For the analysis of (III) the *g*-strains and the statistical weights were optimized, while the principal *g*-values were kept fix from (II). The simulated spectra (red) in (II) result from a superposition of system (1) and (2) in a 1.0:0.56 ratio and in a 1.0:0.81 ratio in (III), respectively.

Figure S4

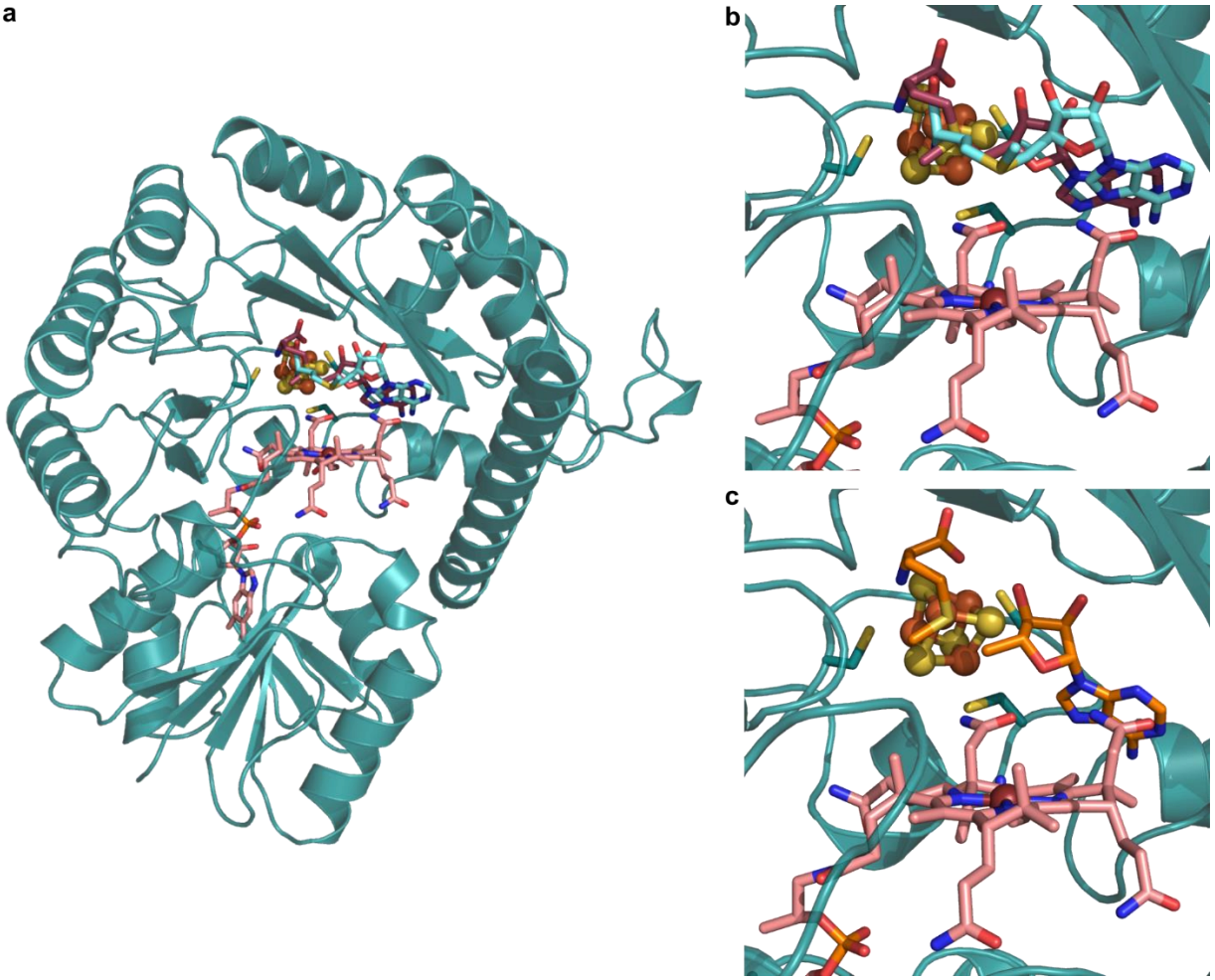

Figure S4. Computational structural model of QCMT from *M. thermophilus* with cofactor arrangement as in OxsB.[7] (a) The structural model of QCMT shown in teal was generated with AlphaFold (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb). The QCMT model was aligned in PyMOL with the crystal structure of OxsB (PDB ID 5UL4), of which the bound cobalamin (rose) and SAM in two distinct orientations (raspberry, cyan) are shown. (b) Focus on the OxsB cofactors placed into the QCMT model. The SAM molecule bound in the typical „Radical SAM conformation" is shown in raspberry, the SAM adopting the potential „methylation conformation" is depicted in cyan. (c) Same as in (b), but with the two SAM molecules replaced by DOA and methionine as observed in the crystal structure of TokK (PDB ID 7KDX).[8]

Figure S5

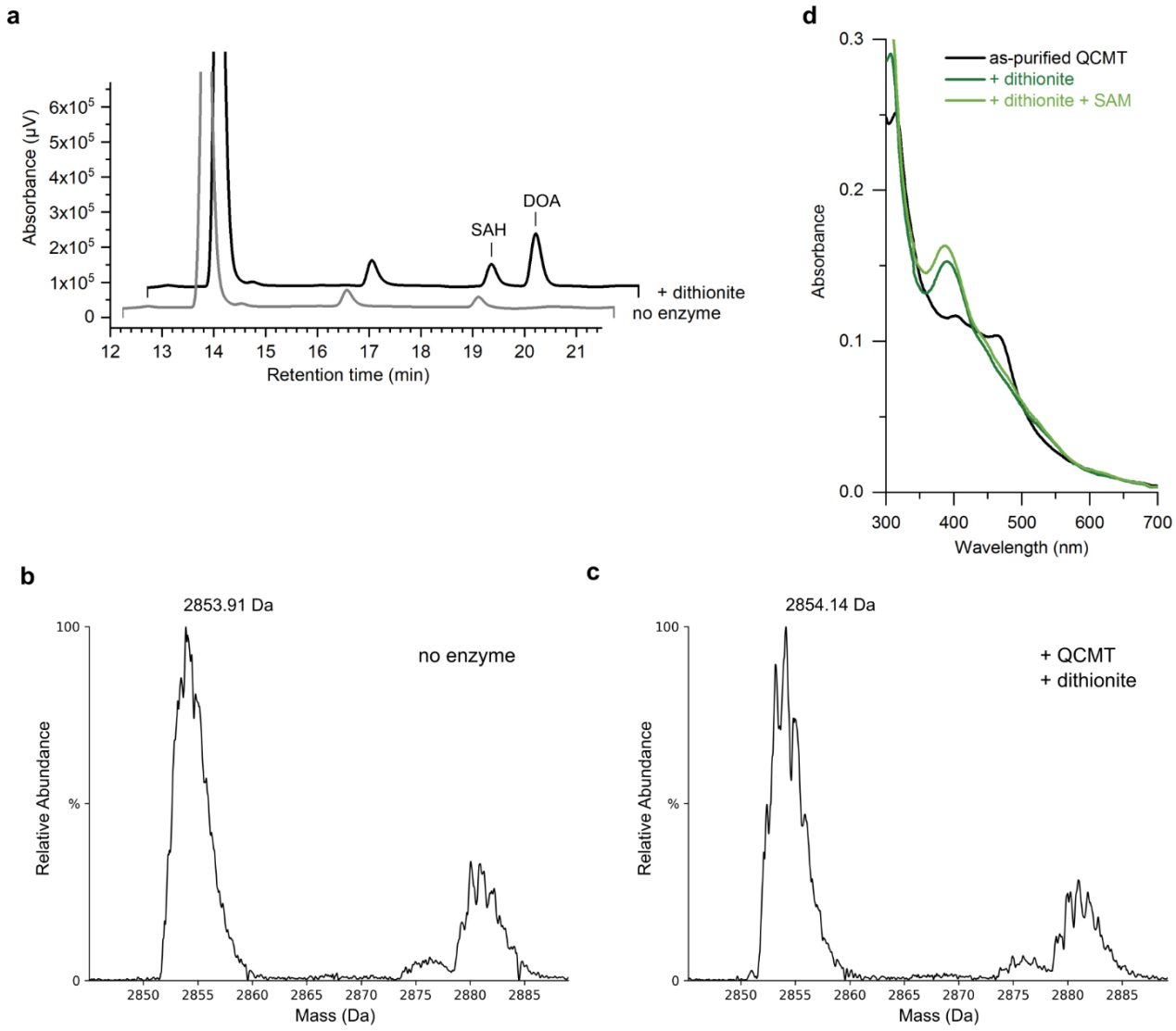

Figure S5. Analysis of QCMT activity with dithionite as reducing agent. (a) HPLC analysis of the initial *in vitro* activity assay mixture containing sodium dithionite (black) and of the initial activity assay without enzyme (grey). (b, c) Deconvoluted EMS LC-MS spectra of peptide QFPTMMEDHFGGSQRAGVIAAASGLSTS from initial QCMT *in vitro* assay containing sodium dithionite. MS-spectra (b) obtained from assay incubations without QCMT enzyme, (c) initial *in vitro* assay incubated with enzyme, showing no methylation for peptide substrate. (d) UV/Vis absorption spectra of as-purified QCMT, as-purified QCMT after addition of dithionite, and as-purified QCMT after addition of dithionite and SAM.

Figure S6

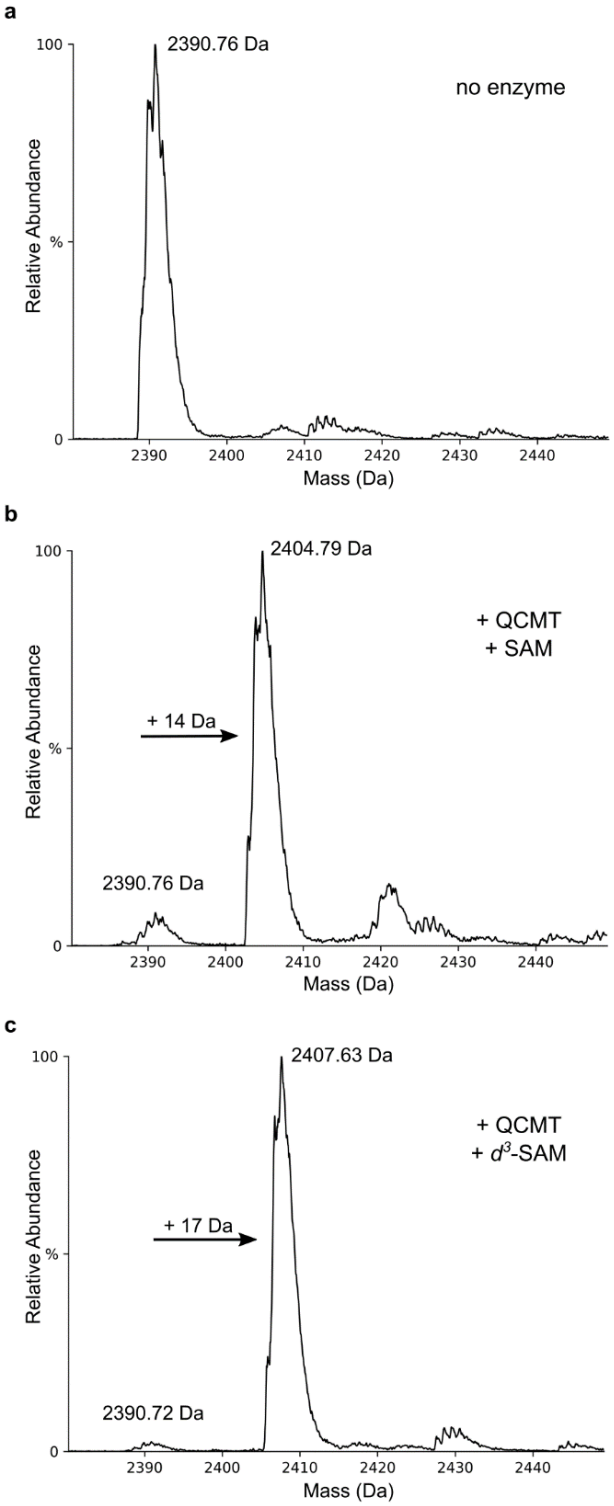

Figure S6. Deconvoluted EMS LC-MS spectra of peptide PTMMEDHFGGSQRAGVIAAASGLS from QCMT *in vitro* assay. (a) MS-spectrum was obtained from assay incubations without QCMT. (b) Peptide methylation by QCMT showing a mass shift of +14 Da. (c) Enzymatic methylation by QCMT with d^3-labeled methionine resulting in a modification with a mass shift of +17 Da.

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Figure S7

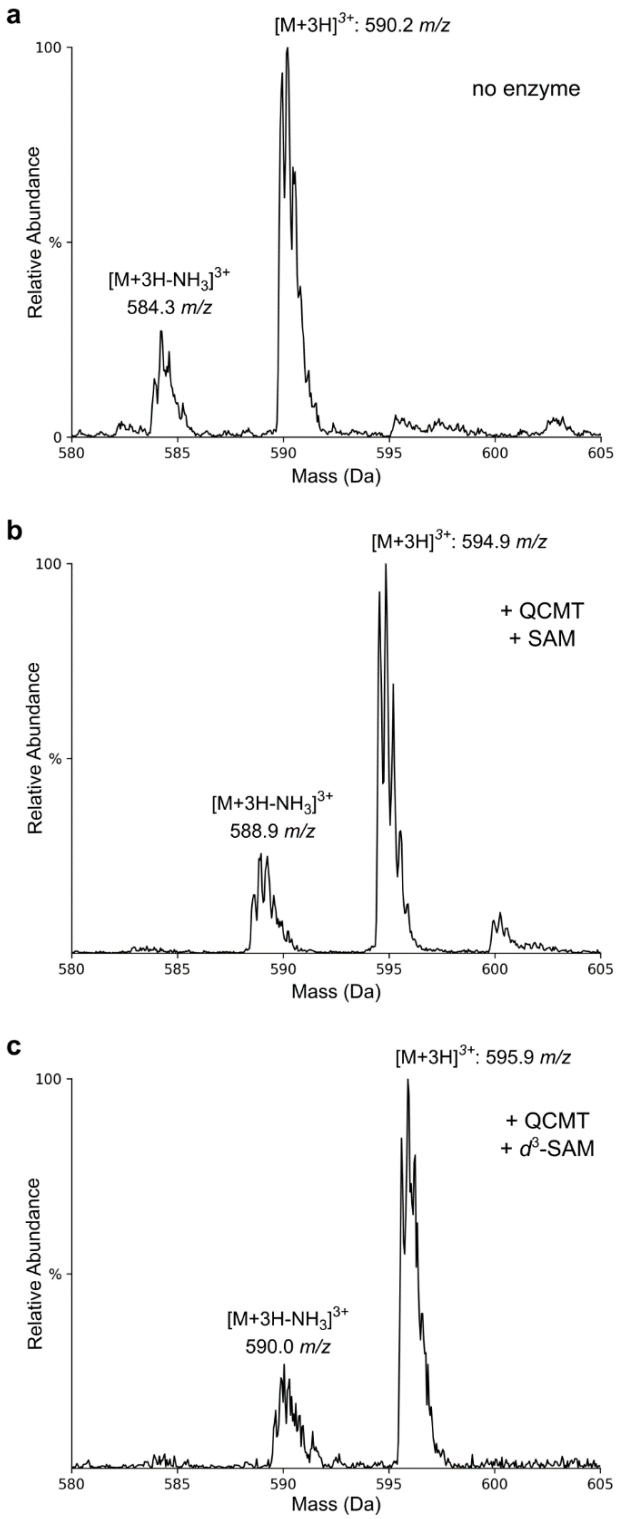

Figure S7. Deconvoluted EMS LC-MS spectra of tryptic peptide QFPTMMEDHFGGSQR from QCMT *in vitro* assay. (a) EMS-spectrum was obtained from an assay mixture without QCMT showing non-methylated precursor: $[M+3H]^{3+}$: 590.2 *m/z* for MS/MS analysis. (b) EMS-spectrum obtained from an assay mixture with QCMT and SAM showing methylated precursor: $[M+3H]^{3+}$: 594.9 *m/z* for MS/MS analysis. (c) EMS-spectrum obtained from an assay mixture with QCMT and d^3-SAM showing d^3-methylated precursor: $[M+3H]^{3+}$: 595.9 *m/z* for MS/MS analysis.

Figure S8

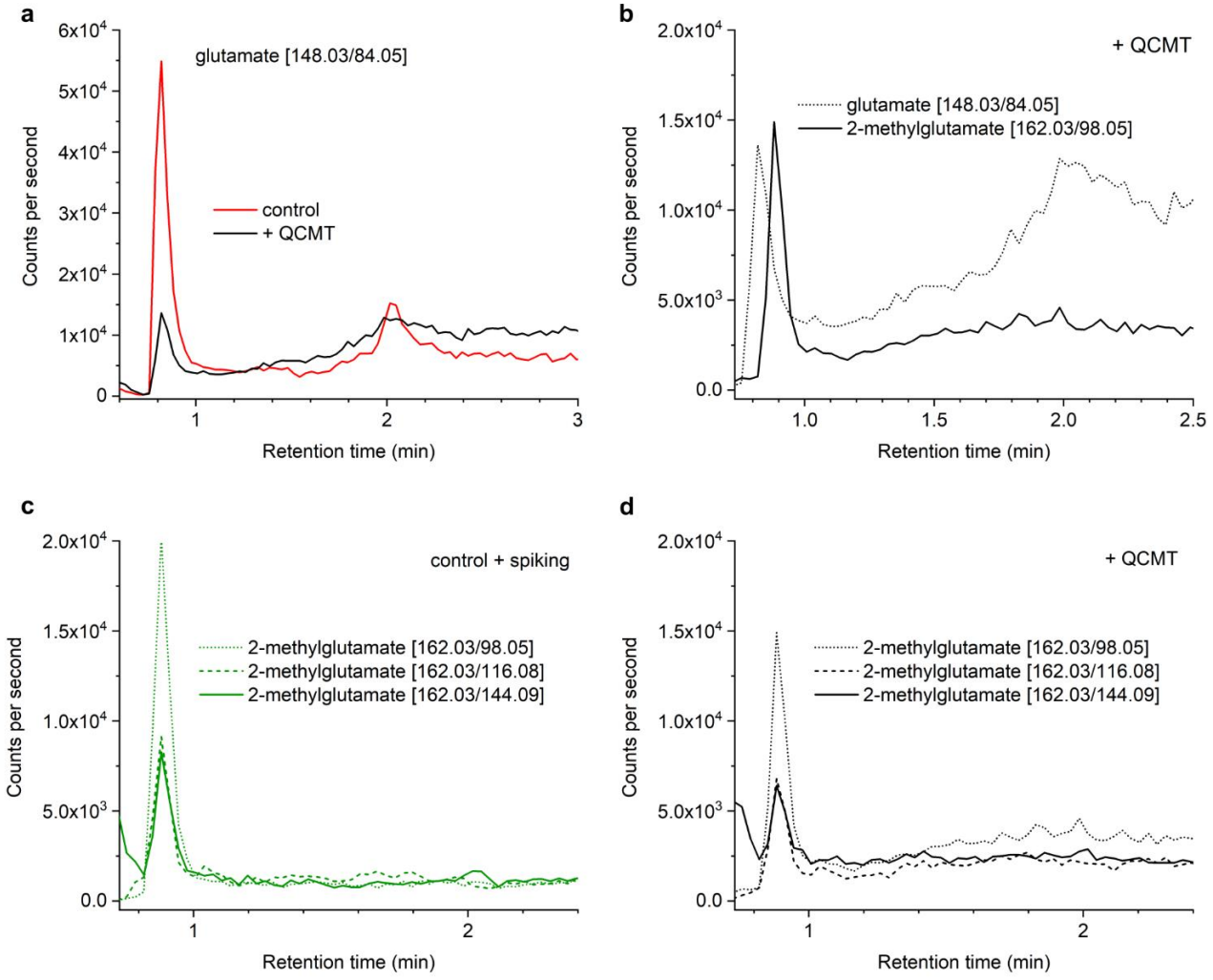

Figure S8. LC-MS MRM data of acid hydrolyzed QS-28 peptide. (a) In the presence of QCMT (black), glutamate is decreased compared to the control lacking enzyme (red). The most abundant MRM transition 148.03/84.05 is shown. (b) In the presence of enzyme, an additional peak (black; MRM transition 162.03/98.05 consistent with 2-methylglutamate) with increased retention time compared to glutamate (grey; MRM transition 148.03/84.05) appears; spiking experiments (Figure 4) confirm its assignment as 2-methylglutamate. Additionally, three independent MRM traces observed for the commercial standard of 2-methylglutamate spiked into the matrix of QS-28 sample lacking enzyme (c) are observed at identical relative intensity levels in the unspiked QS-28 sample in the presence of QCMT (d), consistent with 2-methylglutamate production. Note that during conditions of acid hydrolysis, glutamine is converted to glutamate.

Figure S9

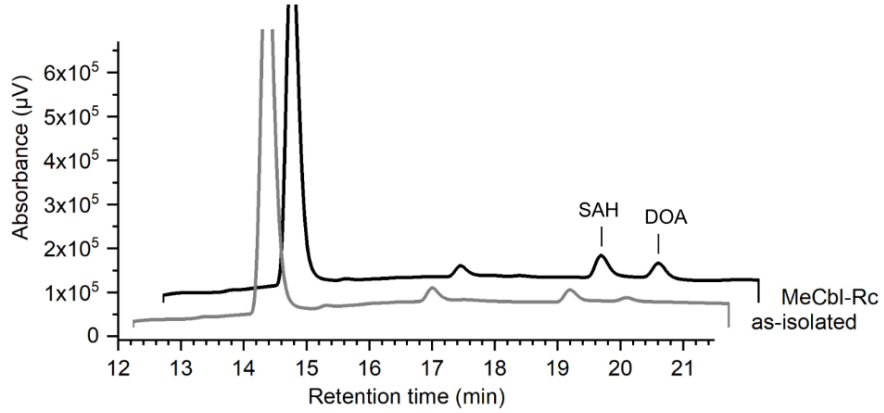

Figure S9. HPLC analysis of the standard *in vitro* activity assay mixture containing as-isolated QCMT (grey) and as-isolated QCMT after reconstitution with methylcobalamin.

Figure S10

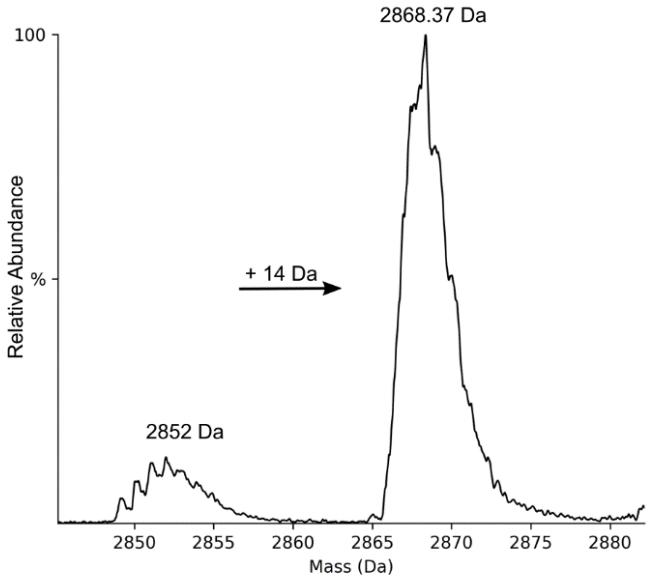

Figure S10. Deconvoluted EMS LC-MS spectra of peptide QFPTMMEDHFGGSQRAGVIAAASGLSTS from QCMT *in vitro* activity assay containing 1 µM QCMT, 200 µM SAM, 50 µM peptide and 1 mM titanium(III) citrate after 6 h of incubation.

Figure S11

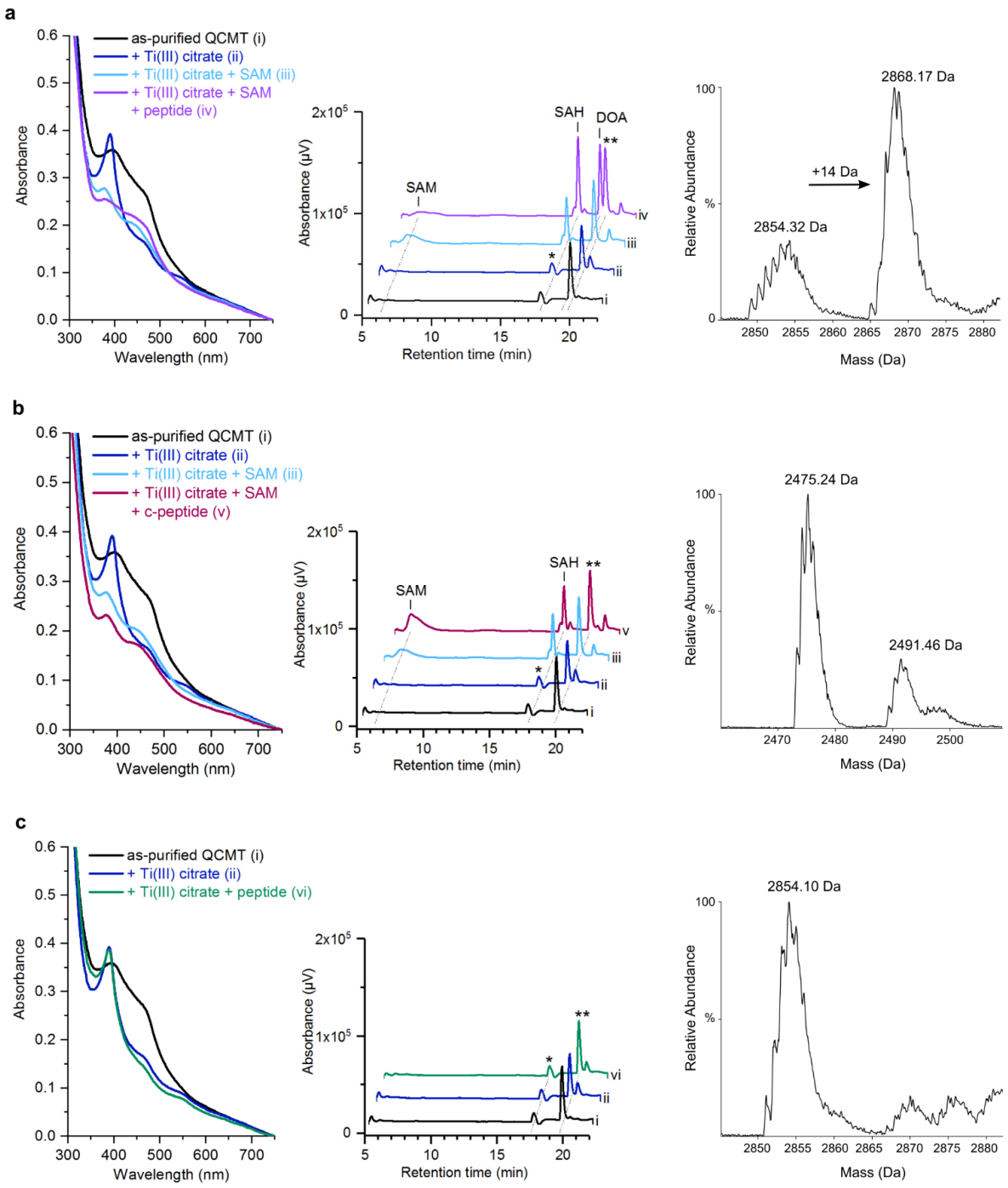

Figure S11. Analysis of QCMT-bound cobalamin and product formation under single turnover conditions. (a) UV/Vis absorption spectra (left) of as-purified QCMT (i), titanium(III) citrate reduced QCMT (ii), titanium(III) citrate reduced QCMT incubated with SAM (iii), titanium(III) citrate reduced QCMT incubated with SAM and peptide substrate (iv). HPLC chromatograms (center) show formation of 10.59 ± 0.13 µM SAH for sample (iii), and 16.66 ± 2.82 µM SAH and 12,15 ± 1.91 µM DOA for sample (iv). The deconvoluted EMS LC-MS spectrum (right) shows peptide methylation after the last step (iv). (b) UV/Vis absorption spectra (left) of samples (i)-(iii) as in (a) as well as titanium(III) citrate reduced QCMT incubated with SAM and a control peptide with a different amino acid sequence (= c-peptide, HAALVSMGEMLPARRARGPNEPGG) (v). HPLC chromatograms (center) show formation of 10.59 ± 0.42 µM SAH but no DOA for sample (v). The deconvoluted EMS LC-MS spectrum (right) shows unmethylated peptide substrate (2475.24 Da) after the last step of incubation (v). The LC-MS spectrum shows additional hydroxylated peptide substrate (2491.46 Da), which occurs under normal storage conditions. (c) UV/Vis absorption spectra (left) of samples (i) and (ii) as in (a) as well as titanium(III) citrate reduced QCMT incubated with peptide substrate (vi). HPLC chromatograms (center) show that neither SAH nor DOA are formed. The deconvoluted EMS LC-MS spectrum (right) shows unmethylated peptide substrate after the last step of incubation (vi).
Concentrations of assay components were: 20 µM as-purified QCMT, 80 µM titanium(III) citrate, 40 µM SAM and 20 µM peptide substrate.
*unidentified substance detected in all samples. **cobalamin liberated from QCMT by acid denaturation.
HPLC analysis was performed after each step of incubation using the Reprosil-Pur ODS-3 column. n = 3 independent activity assays with the same enzyme preparation were performed for quantification of SAH and DOA.
Retention times: SAM:6.3 min, unknown substance*: 17.5 min, SAH: 17.8 min, DOA: 19.4 min, cobalamin**: 19.8 min

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Author Contributions

J.G. planned and performed the majority of biochemical and spectroscopic experiments. J.G. and G.L. analyzed and interpreted biochemical and UV/Vis data. S.J. and C.L. planned and performed LC-MS, analyzed and interpreted MS data. L.G. and J.N.A provided MAT and performed some activity assays for deuterium labeling. T.F. performed EPR spectroscopy, analyzed and interpreted EPR data. L.H. performed the simulation of EPR spectra. G.L. developed the research project. All authors contributed to manuscript writing.