How an Enzyme Binds the C1 Carrier Tetrahydromethanopterin

STRUCTURE OF THE TETRAHYDROMETHANOPTERIN-DEPENDENT FORMALDEHYDE-ACTIVATING ENZYME (Fae) FROM METHYLOBACTERIUM EXTORQUENS AM1**[S]

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The atomic coordinates and structure factors (code for Fae with (1Y60) without (1Y5Y) methylene-H4MPT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: H4F, tetrahydrofolate; Hmd, H2-forming methylene-H4MPT dehydrogenase; Fae, formaldehyde-activating enzyme; MOPS, 4-morpholinepropanesulfonic acid.

Tetrahydromethanopterin (H4MPT) and tetrahydrofolate (H4F) are coenzymes of analogous structure (1) (see Fig. 1). Both coenzymes are involved in the interconversion of C1 units at the oxidation level of formate (1Y5Y) and without (1Y5Y) methylene-H4MPT. Coenzyme binding is accompanied by side chain rearrangements up to 5 Å and leads to a rigidification of the C-terminal arm, a formation of a new hydrophobic cluster, and an inversion of the amide side chain of Gln88. Methylene-H4MPT in Fae shows a characteristic kink between the tetrahydropyrazine and the imidazolidine rings of 70° that is more pronounced than that reported for free methylene-H4MPT in solution (50°).

Methane is likely to depend on H4MPT (9), a process catalyzed by a group of Archaea closely related to the Methanosarcinales (10, 11). All these organisms are highly specialized in C1 metabolism and are of great ecological importance in the global carbon cycle (12, 13). The recent documentation of functional H4MPT-dependent enzymes in the enigmatic bacterial group of Planctomycetes re-opened the debate of the evolution of H4MPT and H4F-dependent enzymes because phylogenetic analysis places the Planctomycetes sequences as distant from the archaeal counterparts as from their proteobacterial counterparts (14).

Functionally the most important difference between H4MPT and H4F is the electron-donating methylene group of H4MPT in position 1c (Fig. 1), which is conjugated to N10 through the aromatic ring, whereas H4F has an electron withdrawing carbonyl group in this position (1, 15). One consequence is that the redox potentials of the N6,N10-methylene-H4MPT/N6,N10-methylene-H4MPT couple (390 mV) and of the N6,N10-methylene-H4MPT/N5,N10-methylene-H4MPT couple (310 mV) are almost 100 mV more negative than the corresponding H4F couples. The structural and functional differences between H4MPT and H4F are reflected in the finding that most of the enzymes catalyzing the interconversion of their C1 derivatives are highly specific for H4MPT or H4F. Their primary structures indicate that most of them have evolved separately (1, 16) although H4MPT and H4F specific enzymes catalyze analogous reactions. This is also true for most of the enzymes involved in the biosynthesis of H4MPT and of H4F (17). The two C1 carriers thus appear to be products of convergent evolution.

The crystal structures of six H4MPT-specific enzymes have been determined and recently reviewed by Shima et al. (18): formylmethanofuran:H4MPT formyltransferase, methenyl-H4MPT cyclohydrolase, F420-dependent methylene-H4MPT...
dehydrogenase (19), NADP-dependent methylene-H4MPT dehydrogenase (20), and F420-dependent methylene-H4MPT reductase. So far none of these enzymes could be crystallized in complex with H4MPT or one of its derivatives. Only the conformation of methylene-H4MPT bound to H2-forming methylene-H4MPT dehydrogenase (Hmd) was determined by two-dimensional NMR spectroscopy (21).

Here we describe the structure of the formaldehyde-activating enzyme Fae from *Methylobacterium extorquens* AM1 with and without methylene-H4MPT bound. The enzyme catalyzes the condensation of formaldehyde with H4MPT to methylene-H4MPT (22). This reaction also proceeds spontaneously but only at a lower rate. Fae was discovered in *M. extorquens* AM1, which grows aerobically at the expense of methanol oxidation to CO2 involving N5,N10-methylene-H4MPT, N5,N10-methenyl-H4MPT, and N5-formyl-H4MPT as intermediates (23). Fae appears to be specific for H4MPT; no formaldehyde-H4F condensing activity could be found with purified Fae (22). Fae minus mutants of *M. extorquens* AM1 are no longer capable of growth on methanol and are inhibited by trace amounts of formaldehyde indicating that Fae has a vital function in methylotrophic energy metabolism and formaldehyde detoxification (22). The importance of Fae for methylotrophy is also reflected by its high abundance in the cytoplasm of the cell (24) and its presence in diverse methylotrophic bacteria (23). Functional orthologs of Fae are also present in some methanogenic Archaea (22) and in *Planctomycetes* species (14). The widespread occurrence of Fae orthologs suggests that formaldehyde may play an unknown but important role in a broad group of prokaryotes.

**EXPERIMENTAL PROCEDURES**

*M. extorquens* AM1 is the strain deposited under DSM 1338 in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

**Heterologous Overproduction of Fae and Purification—Amplification of the fae gene was achieved with Expand-DNA-polymerase (Roche Applied Science), the primers 5'-GAGACCCATATGGCAAAAATCAC-CAAGGTTC-3' (sense, the NdeI site is underlined) and 5'-CTGCCAGGAATTCCTCCGATCTAAGCGTT-3' (antisense, the EcoRI site is underlined), and chromosomal DNA of *M. extorquens* AM1 as a template. The PCR product was digested with NdeI and EcoRI and ligated into the pET17b expression vector and then introduced into *Escherichia coli* BL21 (DE3) pLysS. Each transformant of *E. coli* BL21 (DE3) pLysS was grown aerobically at 37 °C on minimal medium M9 (25) supplemented with ampicillin (100 μg ml⁻¹) and chloramphenicol (50 μg ml⁻¹). When the A600 of the culture reached 0.5, cells were induced by 2 mM isopropyl-β-D-thiogalactopyranoside. After 4 h, the cells were harvested by centrifugation at 4200 × g at 4 °C. Selenomethionine-labeled protein was produced using the method of metabolic inhibition (26).

**Non-labeled Fae and selenomethionine-labeled protein were purified under aerobic conditions as described in Vorholt et al. (22) for Fae from *M. extorquens* AM1. H4MPT was purified from *Methanothermobacter*...
marburgensis (DSM 2133) (27) and stored in 10 mM MOPS/KOH buffer pH 7. H4MPT from *M. extorquens* AM1 differs from that in *M. marburgensis* by lacking the phosphatase and hydrosulfytutarase. It has been shown, however, that enzymes from *M. extorquens* AM1 are equally able with H4MPT and with the dephospho form (22).

**Crystallization and Data Collection**—Crystallization trials were performed with non-labeled and selenomethionine-labeled Fae at 4 °C under aerobic conditions and with enzyme in the presence of H4MPT at 8 °C under strictly anaerobic conditions. Within a hanging drop experiment each drop consisted of 1 μl of enzyme solution (13 mg/ml) and 1 μl of reservoir solution. Crystals of non-labeled Fae as well as selenomethionine-labeled Fae grew in a reservoir solution composed of 0.2 M calcium chloride × 2H2O, 0.1 M sodium acetate × 3H2O, pH 4.6, and 10–20% (v/v) isopropanol. Their space group was P4 3212, and the lattice parameters were a = b = 120.7 Å and c = 205.9 Å. For crystallization in the presence of H4MPT, the enzyme solution (13 mg/ml) was supplemented with 5 mM H4MPT and then combined with the reservoir solution containing 0.1 μM HEPES/NaOH, pH 7.5, and 20% (v/v) polyethylene glycol 10,000. The space group was P21, and the lattice parameters were a = 48.9 Å, b = 112.6 Å, c = 72.0 Å and α, γ = 90°, β = 91°. Data were collected at 4°C at the European Synchrotron Radiation Facility, Grenoble, France (Table I). Processing and scaling were performed with XDS (28) and Denzo/ Scalepack (29).

**Phase Determination and Refinement**—Phases were determined using the multiple anomalous wavelength dispersion method with selenium as anomalous scatterer. The selenium sites were found using SHELXD (30) and further refined using SHARP (31). The phases were calculated with SHARP and improved by solvent flattening (32) assuming a solvent content of 70%. 5-fold molecular averaging within DM (33) resulted in an excellent electron density map where ~80% of the chain could be traced by the automated model-building program MAID (34). Except for 10 residues at the C-terminal end the residual model could be manually incorporated using O (35). Iterative cycles of refinement and manual model building were carried out using the program package CNS (36) and O. The refinement statistics are given in Table I. The structure of the H4MPT-bound enzyme was solved by molecular replacement using the program EPMR (37) with the coordinates of Fae without bound substrate as the search model. After initial refinement the C-terminal amino acids disordered in the coenzyme free structure and H4MPT later replaced by methylene-H4MPT were modeled into the density. The results of the refinement are listed in Table I. The quality of the models was checked with PROCHECK (38).

**RESULTS AND DISCUSSION**

**Structure of Fae with and without Methylene-H4MPT Bound**—Formaldehyde-activating enzyme Fae in the absence and presence of methylene-H4MPT was structurally characterized in two crystal forms at a resolution of 2.0 and 1.9 Å, respectively (Table I). The crystals formed in the presence of H4MPT contained methylene-H4MPT rather than H4MPT, which can be explained by the facts that polyethylene glycols are contaminated with formaldehyde, that methylene-H4MPT forms spontaneously, and enzymatically from formaldehyde and H4MPT under the crystallization conditions.

Fae is organized as homopentameric protein complex with dimensions of about 70 Å × 70 Å × 40 Å (Fig. 2A). Each monomer consists of one compact domain that belongs to the class of α/β proteins. The central sheet contains five strands (β1–β5) joined in the order β1, β2, β3, β4, and β5, with only β4 and β5 oriented in parallel. Helix α1 arranged after strand β2 packs against one side of the sheet; helices α2 (after strand 4) and α3 (after strand 5) pack against the other (Fig. 2B). This architecture is somehow reminiscent to that of the ribosomal protein S5 domain 2-like family to which for example the elongation factor G (39), the ribosomal protein S5 (40), and the galactokinase, homoserine kinase, mevalonate kinase, and phosphomethylate kinase family belong. According to the program DALI (41), the root mean square deviations between Fae and elongation factor G and phosphomethyleate kinase (42) are 2.7 Å and 3.1 Å using ~60% of the Ca positions for calculation. In comparison, the root mean square deviation between the five monomers in the asymmetric unit is ~0.15 Å that between the pentamers of the two crystal forms is 0.6 Å. A rare topological feature of this fold is the β4α2β5 left-handed cross-over linkage that appears to be crucial for the integrity of the fold (Fig. 2B). Compared with the other family members helix α2 in Fae is longer and part of the H4MPT binding site. The major difference between Fae and the other family members is an insertion between strand β2 and β4 (Fig. 2B) consisting of helix α1, strands β3, and an unusual protrusion at the end of strand β3 (see below).

The pentamer can be subdivided into three circular layers built up of an α-helical, a β-sheet, and again an α-helical region (Fig. 2A). The inner ring is formed by the five tightly linked helices α1 of the insertion. The outer layer is formed by helices α2, α3, and α4 with the latter being connected to helix α2 of the next monomer. The central ring consists of the five five-stranded β-sheets with each of them oriented roughly in a perpendicular manner to the neighboring sheet. The hydrophobic core of each sheet is enlarged by helices α1 of the next monomer at the inner side and of the C-terminal segment of the previous monomer at the outer side. A channel crosses the entire pentamer along the 5-fold axis (Fig. 2A) and is occupied with several solvent molecules and extra electron density that could not be assigned.

### Table I

| Data Collection | Fae | Se-methionine | Fae with H4MPT bound |
|-----------------|-----|---------------|---------------------|
| Space group     | P4_2_2 | P4_2_2 | P4_2_2 |
| Completeness (%)| 98.6 (95.4) | 98.6 (98.8) | 99.9 (99.9) |
| R_sym (%)       | 5.4 (40.0) | 6.0 (25) | 6.1 (45.6) |
| b/σ             | 19.3 (2.9) | 17.1 (3.1) | 17.5 (2.1) |
| Redundancy      | 3.5 | 4.6 | 4.0 |
| Refinement statistics |             |             |             |
| No. of residues, solvent and H4MPT molecules | 797, 558, 0 | 841, 492, 5 |             |
| No. of monomers in the asymmetric unit | 5 | 5 |             |
| Resolution range (Å) | 19.89-2.0 | 44.82-1.90 |             |
| Reflections (I > 2σ) | 100903 | 59980 |             |
| R/workings, R_free (%) | 20.4, 23.9 | 20.9, 24.1 |             |
| B_average       | 40.5 | 29.0 |             |
| B_Wilson        | 34.1 | 20.9 |             |
| Root mean square deviation from ideal values | 0.015, 1.6 | 0.011, 1.6 |             |
| Bond lengths (Å), angles (°) |             |             |             |
The binding site for methylene-H₄MPT is located in a 20 Å long, 8 Å wide, and 12 Å deep cleft at the interface between two adjacent subunits called A and B (Fig. 2) with the constituting residues highly conserved (Fig. 2C). Upon methylene-H₄MPT binding the width of the cleft is slightly decreased because of a rotation of helix α2 of about 5° and because of a displacement of strands β1, β2, and β5 in the range of 0.3–0.5 Å. Additionally, the flexible C-terminal arm of subunit A (A160–A166) is rigidified, and the protrusion of subunit B is shifted around 2 Å toward the coenzyme.

Conformation of Methylene-H₄MPT When Bound to Fae—Methylene-H₄MPT binds to the binding cleft with a high occupancy of about 80%. However, the temperature factor increased dramatically from the pterin and imidazolidine rings (30 Å²) via the benzene ring (38 Å²), the ribitol group (50 Å²), the ribose group (68 Å²), to the phosphate group (78 Å²) indicating only an excellent electron density of the functionally relevant head group. The 2-hydroxyglutarate group is located in the bulk solvent (Fig. 3) and is not visible in the electron density map. Note that Fae from *M. extorquens* AM1 was crystallized to-

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**Fig. 2. Structure of formaldehyde-activating enzyme Fae from *M. extorquens* AM1.** A, stereoview of the homopentamer with five methylene-H₄MPT molecules bound when viewed perpendicular to the 5-fold axis. The monomers are shown in red, orange, yellow, green, and blue. B, ribbon diagram of the monomer emphasizing the segment of the αβ fold that is reminiscent to that of the ribosomal protein S5 domain 2-like family in dark and light green, the left-handed ββ cross-over linkage in light green, and the insertion region in red. Fig. 2, A and B, and Figs. 3 and 4 were generated with MOLSCRIPT (46) and RASTER3D (47). C, molecular surface representation of the Fae pentamer highlighting the five methylene-H₄MPT binding clefts and the high degree of conservation of their constituting residues. The surface was colored in blue when the equivalent residues in at least eight of the nine aligned sequences were identical to Fae from *M. extorquens* AM1 (see supplemental data). The figure was generated with GRASP (48).
together with H₄MPT from *M. marburgensis* rather than with the shorter H₄MPT from *M. extorquens* AM1 (7). H₄MPT from *M. marburgensis* contains 11 asymmetric carbons (Fig. 1). The quality of the electron density map allowed us to deduce the stereoconfiguration of five of these, 7α, 6α, 11α, 2c, and 3c (Fig. 1), which agreed with that determined previously by two-dimensional NMR spectroscopy (43).

Methylene-H₄MPT is accommodated into its binding site in an “S”-shaped conformation with the S positioned in a perpendicular manner to the front side of the cleft (Fig. 3). The pterin ring points toward the channel bottom, the imidazolidine ring and the phenyl ring are attached roughly parallel to the length of the cleft, and the ribose and phosphate groups are directed toward the bulk solvent. The S shape of methylene-H₄MPT is the result of two kinks (Fig. 3A). The first sharp kink of ~70° is located between the pterin and the imidazolidine rings around the N⁵-C⁶a bond. An additional small rotation between the imidazolidine and the phenyl rings results in a nearly perpendicular orientation between the pterin and the phenyl ring. The second kink of roughly 90° is performed within the ribitol group. This conformation of methylene-H₄MPT implies that solely the pterin ring is shielded from bulk solvent by the described roof. The rest of the methylene-H₄MPT including the imidazolidine ring is at least partly solvent accessible.

The conformation of methylene-H₄MPT in the Fae-methylene-H₄MPT complex (as described by this work) is different from the conformations of methylene-H₄MPT in solution or when bound to Hmd, which have previously been determined by two-dimensional NMR spectroscopy (21). The major conformational surprise of methylene-H₄MPT in Fae is the large

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**Fig. 3.** Protein-methylene-H₄MPT interactions. A, stereoview of the entire methylene-H₄MPT binding site showing all relevant contacting residues. B, stereoview focused on the methyl groups 12a and 13a of methylene-H₄MPT and the C-terminal arm. The inclusion of some residues of the empty enzyme (blue) illustrates the induced fit movement of the enzyme (gray) upon coenzyme binding and the importance of the C-terminal arm for binding. The model is depicted in a ball-and-stick representation. White, carbons of methylene-H₄MPT; light gray, carbons of the polypeptide of subunit A; gray, carbons of the polypeptide of subunit B; red, oxygen; blue, nitrogen; yellow, phosphorus.
when bound to Hmd. Consequently, atom C7a of methylene-tetrahydropyrazine ring. The C7a atom has to point to the Si face in Fae but to the Re face in solution and when bound to Hmd. Consequently, atom C7a of methylene-H4MPT bound to Fae is oriented to the Re face that leads to a conformation of the C13a atom perpendicular to the pterin ring, whereas the Si face orientation leads to an equatorial position as found in the free and Hmd bound form (Fig. 4). Obviously, the protein scaffold of Fae substantially influences the conformation of methylene-H4MPT upon binding. For example, methylene-H4MPT in the conformation found in solution would interfere with Pheα166 of Fae that could not evade because of its contact to Valβ81. A related conformational variability of the kink is expected for methylene-H4F dependent enzymes, although an enzyme-methylene-H4F complex is so far not structurally characterized.

Interactions between Fae and Methylene-H4MPT—The increasing flexibility along the elongated molecule is reflected in a parallel decrease of the protein-cofactor interactions. Only two hydrogen bonds and a few van der Waals contacts are formed between the protein matrix and the tail groups of H4MPT (Fig. 3A). In other words binding is essentially based on interactions between the polypeptide chain and the catalytically relevant head groups. The conserved residues Aspα24, Lysα71, Leuβ53, and Glnβ88 form specific hydrogen bond interactions toward the N1, NH2a, N3H, and O4a atoms (Fig. 3A). The head groups including the imidazole group of Hisα22 are mainly interact with invariant hydrophobic side chains of the C-terminal arm (Fig. 3B).

![Fig. 4. Conformational diversity of methylene-H4MPT. Structural alignment of methylene-H4MPT bound to Fae (black), to H2-forming methylene-H4MPT dehydrogenase (green), and in solution (red). The superposition is based on the pyrimidine ring and atoms N5 and N6 of the piperidine ring.](image)

Interestingly, the binding site of methylene-H4MPT cannot be considered as prebuilt. In the empty enzyme Pheα119, Pheβ84 and Hisα22 point into the coenzyme binding site but evade the arriving methylene-H4MPT by movements up to 5 Å thereby inducing many additional conformational changes. For example, the rotation of Pheα119 induces substantial rearrangements of the side chains of Hisα121 and Gluα123 and allows Lysα71 to partially fill out the generated free place. Likewise, Pheβ84 swings toward the channel bottom accompanied by a shift of Leuβ52 to the Re side of the pterin ring that induces a movement of Thrβ50 value of 1.9 Å. Consequently, a modeling of methylene-H4MPT to the empty Fae structure would not be possible.

The mentioned residues are conserved in Fae from different organisms but not in any of the other H4MPT-specific enzymes. A common binding motif for the C1 carrier was not found. This was also not to be expected because the known H4MPT specific enzymes are not similar on the sequence nor on the structural level. This also holds true for the different H4F specific enzymes, which also do not show a common H4F binding motif.

Selectivity of Fae for Methylene-H4MPT Rather Than for Methylene-H4F—Fae catalyzes the reaction of formaldehyde with H4MPT. Using the same assay a formation of methylene-H4F from formaldehyde and H4F was not observed (22). The specificity of Fae for H4MPT is also indicated by the finding that the rate of condensation of formaldehyde and H4MPT was not inhibited by the addition of an excess of H4F.2

This finding needs discussion because except for two methyl groups H4MPT and H4F only differ in their tail groups, but as described the ribitol, ribose, and phosphate groups of methylene-H4MPT appear to contribute only slightly to binding (Fig. 3). Although these few interactions as well as an interference between the protein and the formylglutamate tail groups in H4F might be crucial for selectivity the structural data support

![Fig. 5. Proposed mechanism of methylene-H4MPT formation from formaldehyde and H4MPT. The reaction can be subdivided in a nucleophilic addition and a nucleophilic substitution process. A key function in formaldehyde activation and catalysis is attributed to Hisα22, which is strictly conserved.](image)
a binding mechanism that attributes a key function to the additional methyl groups (Fig. 3B). Accordingly, three of four van der Waals contacts between the methyl groups are formed to side chains of the C-terminal arm that might contribute to its partial fixation. A simultaneous conformational change of several side chains at the Re side of methylene-H$_4$MPT generates a hydrophobic cluster composed of the tetrahydroprazine and the imidazolidine rings, Leu$_{A}^{113}$, His$_{A}^{164}$, Pro$_{A}^{165}$, Phe$_{A}^{166}$, and Pro$_{B}^{558}$ and most interestingly triggers an inversion of the amide group of Glu$_{A}^{488}$. This exchange of the amide oxygen and amine groups is the prerequisite to form two hydrogen bonds to methylene-H$_4$MPT and one hydrogen bond to His$_{A}^{164}$ of the C-terminal arm (Fig. 3B). Thus, preferred binding of H$_4$MPT against H$_4$F is not only accomplished by the quantitatively small van der Waals interactions between the methyl groups and the protein but by an induced cooperative process that enhances both methylene-H$_4$MPT binding and the fixation of the C-terminal arm. The interactions between the C-terminal arm and the methyl groups of methylene-H$_4$MPT might additionally influence the kink angle between the pterin and imidazolidine rings and thus the binding energy between methylene-H$_4$MPT and Fae.

Formaldehyde Binding Site and Enzymatic Mechanism—Attempts to determine a structure of Fae in complex with formaldehyde failed, but an attractive binding site of the substrate is offered by the structure of the Fae-methylene-H$_4$MPT complex. At first glance, an activation of formaldehyde by the amine group of Lys$_{A}^{171}$ appears to be chemically plausible (Fig. 3A), but its fixation by a large number of interactions and the absence of space for formaldehyde binding without pushing H$_4$MPT out of its binding site reject this possibility. More attractively, a site either occupied with a solvent molecule or an unknown molecule (depending on the considered cleft of the asymmetric unit) is positioned parallel to the imidazolidine ring and is accessible from bulk solvent. Furthermore, the oxygen atom of formaldehyde can be modeled into the site of the solvent molecule or into a protrusion of the electron density of the unknown molecule and the methyl groups of methylene-H$_4$MPT might additionally influence the kink angle between the pterin and imidazolidine rings and thus the binding energy between methylene-H$_4$MPT and Fae.

In agreement with the results of kinetic experiments of spontaneous methylene-H$_4$F formation from H$_4$F and formaldehyde (44). The spontaneous reaction proceeds optimally under acidic conditions indicating that a protonation step is involved. Most likely, in the enzyme, the proton for this step is provided by protonated His$_{A}^{22}$. Thus, the presented structure of the Fae-methylene-H$_4$MPT complex is not only the prototype of how this cofactor binds to an enzyme, but it also provides insights into the mechanism of how the highly toxic intermediate formaldehyde is metabolized and thus detoxified.

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