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Crystallization and preliminary X-ray analysis of the formiminotransferase domain from the bifunctional enzyme formiminotransferase–cyclodeaminase

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Formiminotransferase–cyclodeaminase (E.C. 2.1.2.5–E.C. 4.3.1.4) is a bifunctional enzyme involved in the histidine-degradation pathway which exhibits specificity for polyglutamylated folate substrates. The first function of the enzyme transfers the formimino group of formiminoglutamate to the N^5 position of tetrahydrofolate, while the second function catalyses the cyclodeamination of the formimino group, yielding $N^{5,10}$-methenyl-tetrahydrofolate, with efficient channeling of the intermediate between these activities. Initial studies have shown that the enzyme consists of eight identical subunits of 62 kDa each, arranged as a circular tetramer of dimers. It is this formation which results in two different dimeric interfaces, which are necessary for the two different activities. The identical subunits have been shown to consist of two domains, each of which can be obtained as dimers. The formiminotransferase domain has been crystallized in the presence of the substrate analogue folinic acid. The crystals belong to space group $P2_12_12_1$, with unit-cell dimensions a = 64.4, b = 103.7, c = 122.3 Å. Both a native data set and a mercurial derivative data set have been collected to 2.8 Å resolution.

1. Introduction

The bifunctional enzyme formiminotransferase–cyclodeaminase (FTCD) catalyses two independent sequential reactions in the histidine-degradation pathway in mammalian liver. The transferase activity of FTCD transfers the formimino group of formiminoglutamate to the N^5 position of tetrahydrofolate, producing 5-formiminotetrahydrofolate and glutamate. The cyclodeaminase activity catalyses the cyclization of the formimino group yielding $N^{5,10}$-methenyl-tetrahydrofolate and releasing ammonia (see Fig. 1). By transferring a one-carbon unit from formiminoglutamate to tetrahydrofolate, these enzymatic reactions serve as an additional contribution to the one-carbon tetrahydrofolate pool.

FTCD has been determined to consist of eight identical subunits arranged to form a circular octamer (Beaudet & MacKenzie, 1975; MacKenzie *et al.*, 1980). It comprises eight identical subunits of 62 kDa each, which are arranged in an octamer, more accurately described as a tetramer of dimers, which results in the formation of two different subunit interfaces. Dissociation and renaturation studies of FTCD suggested that one dimeric interface is responsible for the transferase activity and the other for the cyclodeaminase activity (Findlay & MacKenzie, 1988). Further evidence supporting this finding was provided by deletion mutagenesis (Murley & MacKenzie, 1995). These studies showed that each subunit consists of a N-terminal transferase active domain and a C-terminal deaminase active domain which are separated by a short linker sequence. Expression and purification showed that each domain exists as a monofunctional dimer (Murley & MacKenzie, 1995).

Binding studies demonstrated that there are four polyglutamate binding sites per octamer (Paquin *et al.*, 1985) and that the polyglutamate-binding specificity resides in the deaminase domain (Knighton *et al.*, 1994). FTCD can channel polyglutamylated formiminotetrahydrofolate between the transferase and deaminase active sites (MacKenzie, 1979; MacKenzie *et al.*, 1980). Substrate channeling is a kinetic process by which a metabolite or intermediate is directly transferred from one enzyme active site to another site distanced from the first without being released from the enzyme into solution. This process increases the efficiency in product transfer between active sites within an enzymatic reaction pathway and thus increases the rate of the overall reaction. Two different molecular mechanisms have been observed for channeling. In the enzyme tryptophan synthase, a 25 Å long hydrophobic tunnel has been observed to channel the hydrophobic intermediate indole between the two active sites (Hyde *et al.*, 1988). In contrast to a buried tunnel, the enzyme thymidylate synthase–dihydrofolate reductase from *Leishmania*

major reveals a large positive electrostatic surface straddling the 40 Å distance between the two active sites, which is believed to allow channeling of the hydrophilic polyglutamate form of the substrate between the active sites (Knighton *et al.*, 1994). Formiminotransferase–cyclodeaminase also uses a non-covalently linked polyglutamate form of the substrate; however, this protein has a much greater specificity for the length of the polyglutamate chain than has been observed for thymidylate synthase–dihydrofolate reductase, and optimal channeling is observed with the pentaglutamate form of the substrate (MacKenzie, 1979). The chain-length specificity led to the postulate that the polyglutamate chain acts to anchor the substrate to the octamer, thereby allowing the substrate to move optimally between active sites (MacKenzie & Baugh, 1980).

There are a number of advantages for enzymes to express two distinct catalytic functions in a single gene and to channel the intermediate between the two active sites. In the case of tryptophan synthase, channeling provides a useful means by which the non-polar intermediate can be retained with minimal loss by escape through the cell membrane. In the case of thymidylate synthase–dihydrofolate reductase, the binding of the substrate to the enzyme through a polyglutamyl group may prevent the loss of the intermediate by diffusion through the solution. Channeling could be advantageous to formiminotransferase–cyclodeaminase, since the formiminotransferase reaction produces a labile intermediate which would degrade upon release to the surrounding solution before the second reaction could be completed.

In order to further understand the molecular details of channeling by this bifunctional enzyme and to characterize the distinct catalytic mechanisms, we have undertaken crystallographic studies of FTCD. Here, we report crystals of the formiminotransferase domain in the presence of the bound substrate analogue folinic acid. In addition, we outline preliminary heavy-atom data statistics.

2. Results and discussion

2.1. Protein purification and crystallization

Hexahistidine-tagged formiminotransferase domain was overexpressed using the pBKe-Cm1 expression vector in *Escherichia coli* strain BL21/DE3 and purified to homogeneity as described previously (Murley & MacKenzie, 1995), omitting the last DEAE Sepharose column. The pooled fractions containing the FT domain were then dialyzed into 25 m*M* MOPS pH 8.2, 10 m*M* K_2SO_4 pH 7.3, 35 m*M* β-mercaptoethanol and 10%(*v*/*v*) glycerol, with the addition of 20 m*M* EDTA to remove any Ni^{2+} which may have leached from the Ni-NTA column. The protein was dialyzed again in the same buffer excluding EDTA to remove any chelated Ni^{2+} and EDTA. It was then concentrated to 8 mg ml^{-1} using a Centriprep 10 and Centricon 3 (Amicon Inc.). Protein concentrations were determined using a Bradford assay with BSA as the standard.

Crystallization conditions were screened by the hanging-drop vapour-diffusion technique. Initial trials using a sparse-matrix screen, described by Jancarik & Kim (1991), showed a promising granular precipitate with some precipitants (ammonium sulfate, sodium formate, sodium citrate, polyethylene glycol 2000 and 8000). Further crystallization experiments around these conditions did not result in any significant improvement. It is well known that many proteins have considerable conformational flexibility. This flexibility is a source of structural heterogeneity which may act to inhibit crystallization. Structural heterogeneity is particularly prevalent when one is dealing with multidomain proteins where the interdomain contacts may be rather flexible (Sousa, 1997). It was thought that introduction of a ligand to the crystallization trials of FT domain might act to enhance crystal formation, particularly if such conformational flexibility exists. The ligand may act to 'lock' the protein in a single conformational state, thus rendering it more amenable to crystallization. For this reason, we decided to screen crystallization conditions of FT domain with various substrates and substrate analogues. In particular, the substrate analogues 2 m*M* folinic acid and 2 m*M* glutamate were added, either together or independently, to the conditions from the random screening which gave the most granular precipitates. X-ray diffraction quality crystals were obtained using 1 *M* sodium citrate, 100 m*M* Tris pH 8.0 and 15%(*v*/*v*) glycerol as mother liquor with the addition of 2 m*M* folinic acid to the protein buffer described above. Equal volumes of mother liquor and protein were mixed in a drop and the trays were incubated at 290 K for two weeks, after which crystals with a

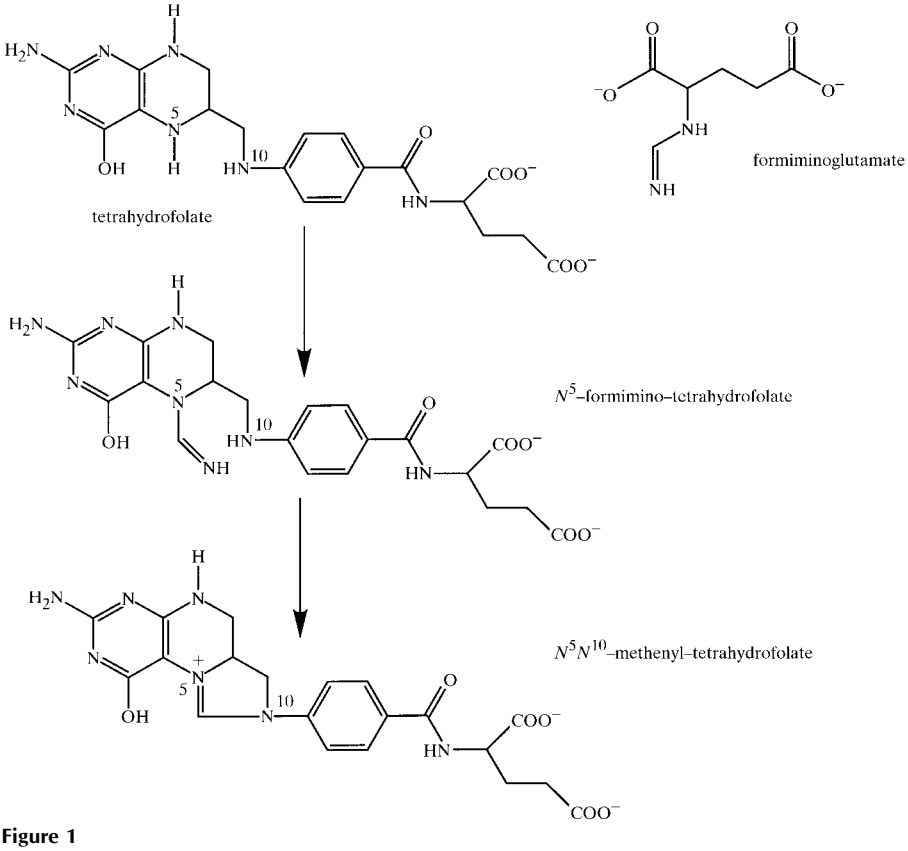

Figure 1
The reaction catalyzed by the bifunctional enzyme, formiminotransferase-cyclodeamainase. The first step involves the transfer of a formimino group from formiminoglutamate to N^5 of tetrahydrofolate and is catalyzed by the formiminotransferase domain of the enzyme. The second step is catalyzed by the cyclodeaminase domain and involves the cyclization of N^5-formimino-tetrahydrofolate to produce $N^{5,10}$-methenyl-tetrahydrofolate.

Table 1
Data collection and processing statistics.

Data set	Resolution (Å)	Total reflections	Independent reflections	Completeness (%)	I/σ	R_{merge}† (%)	R_{deriv}‡ (%)
Native	2.8	110896	20598	98.7	29.0	4.6	
PCMBS	2.8	71107	20197	96.2	10.8	6.5	15.7

† $R_{merge} = \sum\sum|I - \langle I\rangle|/\sum I$ (summed over all intensities). ‡ $R_{deriv} = \sum|F_{deriv_h} - F_{nat_h}|/\sum F_{nat_h}$ (in the resolution range 40–2.8 Å).

typical size of 0.7 × 0.25 × 0.25 mm and a fin-like morphology appeared (Fig. 2).

2.2. Data collection and preliminary X-ray analysis

Prolonged exposure of crystals mounted in a glass capillary tube to the X-ray beam at room temperature revealed significant sample decay. To avoid this decay during data collection, crystals were briefly transferred into a drop of the cryoprotectant, Paratone 8277 (Exxon Oil), mounted in a rayon loop (Hampton Research Co.) and transferred to a stream of nitrogen at 80 K generated by an ADSC (Area Detector Systems Corporation) low-temperature crystal-cooling system. X-ray diffraction data to 2.8 Å resolution were collected on frozen crystals using an imaging-plate detector (MAR Research) mounted on a Rigaku RU-200 rotating-anode X-ray generator (Cu $K\alpha$ radiation). A double-focusing mirror system (Supper Ltd.) was used to produce a highly focused X-ray beam.

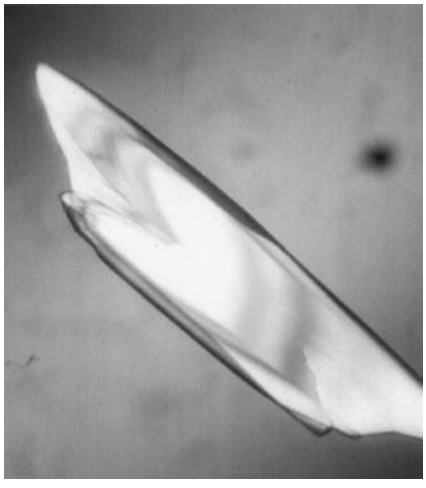

Figure 2
A crystal of formiminotransferase domain grown by vapor diffusion using the hanging-drop method. The crystal size is approximately 0.7 × 0.25 × 0.25 mm.

Data from one native and one heavy-atom derivative have been collected, processed and scaled using the *HKL* software (Minor, 1993; Otwinowski, 1993). Autoindexing revealed that the crystals belong to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions a = 64.4, b = 103.7, c = 122.3 Å. Data analyses were carried out using the *CCP*4 suite of computer programs (Collaborative Computational Project, Number 4, 1994). The data collection and processing statistics are given in Table 1. Using a molecular mass of 74 kDa (for the homodimer) and assuming the presence of one dimer per asymmetric unit, a V_M value of 2.95 Å^3 Da^{-1} was obtained. This value falls within the range observed by Matthews (1968) and corresponds to a solvent content of 50%.

3. Heavy-atom derivatives

Multiple isomorphous replacement (MIR) will be used to solve the phase problem. To this end, a heavy-atom derivative was prepared by soaking crystals in a solution of mother liquor with 1 m*M* *p*-chloromercuribenzenesulfonic acid (PCMBS) for 4 h. Data were collected and processed identically to that for the native (Table 1). Two Hg-atom positions were identified using the Patterson vector superposition method in *SHELXS* (Sheldrick *et al.*, 1993). These heavy-atom parameters for these two positions were refined using the program *MLPHARE* from the *CCP*4 suite. Initial phase estimates revealed that the mercurial derivative is weakly occupied, as seen by the phasing power of 0.91 and the R_{cullis} of 0.79. A search for other derivatives is currently under way, with the expectation that one or two further derivatives with a greater occupancy in combination with solvent flattening and twofold averaging will yield an interpretable electron-density map.

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