SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray analysis of recombinant glutamate mutase and of the isolated component S from *Clostridium cochlearium*

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Abstract

Glutamate mutase $[\varepsilon_2\sigma_2(B_{12})_1]$ was reconstituted by incubating purified components E (ε_2) and S (σ_2) from *Clostridium cochlearium*, both produced in *Escherichia coli*, with either aquo- or cyanocobalamin. The inactive glutamate mutase obtained was crystallized with polyethyleneglycol 4000 as precipitant. Crystals are monoclinic with space group $P2_1$ and have cell dimensions a = 64.6, b = 113.2, c = 108.4 Å and β = 96.0° for the glutamate mutase reconstituted with aquocobalamin. They diffract to a resolution of at least 2.7 Å. Isolated component S was crystallized in the presence of an excess of cyanocobalamin, yielding red crystals of space group $I422$ with unit-cell dimensions of $a = b$ = 69.9 and c = 107.1 Å. The crystals diffract to about 3.2 Å resolution. Native data sets were collected for both crystal forms.

1. Introduction

While at least a ten different B_{12}-dependent enzymes have been identified to date (Pratt, 1993) three-dimensional structural information is available for only two, *i.e.* for the cobalamin binding domain of methionine synthase from *Escherichia coli* (Drennan *et al.*, 1994) and for methylmalonyl CoA mutase from *Propionibacterium shermanii* (Mancia *et al.*, 1996). Although both of these enzymes use cofactors with Co—C bonds, methylcobalamin and adenosylcobalamin (coenzyme B_{12}), respectively, they have strikingly different enzymatic mechanisms. The methyl transfer mediated by methionine synthase involves heterolysis of the Co—C bond, whereas homolysis has been observed in the carbon skeleton rearrangement catalyzed by methylmalonyl-CoA mutase or glutamate mutase (for a recent review see Golding & Buckel, 1997).

Glutamate mutase from *Clostridium cochlearium*, which equilibrates (*S*)-glutamate with (2*S*,3*S*)-3-methylaspartate, has been characterized as a stable heterotetramer ($\varepsilon_2\sigma_2$) containing one coenzyme B_{12}. Although the natural cofactor has been identified as pseudocoenzyme B_{12}, in which the axial base dimethylbenzimidazole has been replaced by adenine (Barker *et al.*, 1958; Friedrich, 1975; Hoffmann & Bothe, 1997 unpublished results), the enzyme also shows high activity with coenzyme B_{12} as cofactor. This is not surprising, since it has been demonstrated that in the active complex, the conserved histidine residue 16 of the σ polypeptide, rather than dimethylbenzimidazole, is coordinated to the cobalt. The genes coding for the polypeptides have been cloned and overexpressed separately in *E. coli*. Upon purification, polypeptide σ (M_r = 14.7 kDa), which has been designed as component S, is obtained as a monomer, whereas the other polypeptide forms a dimer (ε_2, M_r = 107 kDa) and was called component E (Zelder *et al.*, 1994*a*,*b*, 1995).

Component S shows sequence similarity to the B_{12} binding domain S of methionine synthase and methylmalonyl CoA mutase, as has been shown for the case of the related glutamate mutase from *C. tetanomorphum* (Marsh & Hollaway, 1992). This provides strong evidence (which is also supported by the present crystallographic results) that the B_{12} cofactor is bound to the component S. We embarked upon a comprehensive crystallographic investigation of the glutamate mutase from *C. cochlearium* and of its isolated subunits for the following reasons.

(1) The first step of the reaction cycle is a substrate-induced homolysis of the cofactor's organometallic bond. A long-standing question concerns the way substrate binding induces this bond homolysis. The recently published crystal structure of methylmalonyl CoA mutase (Mancia *et al.*, 1996) suggested a possible mechanism of this intriguing process *via* stretching of the trans-annular Co—N bond. It will be of interest to verify such a mechanism for the case of the related glutamate mutase.

(2) Three-dimensional structural data should yield an insight into the steric course of the rearrangement steps following initial formation of the coenzyme B_{12}-derived adenosyl radical. A recent suggestion for the mechanism of this rearrangement process involves fragmentation of the substrate-derived radical (Buckel & Golding, 1996).

We have chosen to start with protein reconstituted with cobalamin derivatives known to be incapable of Co—C bound homolysis under normal conditions. Experience with the related methylmalonyl CoA mutase has shown that when using active enzyme with adenosylcobalamin as cofactor, the labile Co—C bond is easily broken during crystallization and/or diffraction data collection. This may leave the corrinoid cofactor in a poorly defined state. However, we plan to perform experiments with active glutamate mutase once the structure of the inactive variant is known.

2. Purification of the component E and S and recombination of the glutamate mutase

2.1. *Expression of glmS and glmE*

E. coli strain MC 4100 containing the expression vector pOZ3 (Zelder *et al.*, 1994*a*) and *E. coli* strain DH5α containing pOZ5 (Zelder *et al.*, 1994*b*) were used for overproduction of glutamate mutase components S and E, respectively. The bacteria were grown to $OD_{578} \simeq 1$ on Standard 1 nutrient broth (Merck, Darmstadt), induced overnight with 1 m*M*

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isopropyl-1-thio-β-D-galactoside (IPTG) and harvested by centrifugation.

2.2. *Purification of components E and S*

Enzyme activity was measured using the spectrophotometric assay developed by Barker *et al.* (1964). For purification of recombinant component E. the harvested *E. coli* cells were resuspended in buffer *A* (20 m*M* potassium phosphate pH 7.4, 1 m*M* EDTA) and sonicated for 10 min followed by centrifugation. The supernant was applied to a phenyl-Sepharose column (Hiload 26/10, Pharmacia) previously equilibrated with buffer *B* (buffer *A* containing 1 *M* ammonium sulfate). A decreasing linear ammonium sulfate gradient (buffer *B* to buffer *A*, 160 ml) was used to remove *E. coli* proteins. Component E was eluted with water and concentrated to less than 10 ml using Millipore Ultrafree-15 filter units with Biomax-5K membranes (Sigma). The concentrate was applied to a Superdex 200 column and eluted with buffer *A*. Recombinant component S was purified as described for component E, with the exception that component S was eluted at *ca* 0.2 *M* ammonium sulfate from the phenyl-Sepharose column (ammonium sulfate gradient: buffer *B* to 80% buffer *A*, 100 ml, followed by 100% buffer *A*, 300 ml). To buffers *A* and *B*, 2 m*M* dithiothreitol (DTT) was added to avoid oxidative aggregation. The purification procedures were monitored by sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS–PAGE), indicating a purity of at least 95%.

For the recombination of the inactive glutamate mutase, component E was incubated at 310 K for 10 min with three times molar excess of component S and six times molar excess of cobalamin. The excess of component S was essential in order to avoid the contamination of the recombinant inactive enzyme by homodimer component E. After incubation the sample was applied to a size-exclusion chromatography column (Pharmacia) and the purity of the red glutamate mutase containing fractions was monitored by PAGE in the absence and presence of SDS, the purity of the intact protein complex was estimated at 97%.

For crystallization of component S containing cobalamin, the protein was purified as described above, followed by anionic ion-exchange chromatography (Mono Q). Subsequently, it was mixed at room temperature with a threefold molar excess of cobalamin and used for crystallization. The purity of component S was estimated to be 98%.

3. Crystallization

Crystals were obtained with the hanging-drop technique, typically using drops of 2 µl protein (14 mg ml^{-1}) solution mixed with 2 µl reservoir solution. To prevent oxidative aggregation, all solutions contained 1 m*M* DTT and reservoirs were flushed with argon. All crystals were thin platelets, with a very pronounced tendency for twinning. We tried a large variety of conditions and techniques (*e.g.* gel crystallization, sitting drop, microbatch, dialysis, seeding) to improve and optimize crystal size and quality. So far, the best crystals were obtained at room temperature from hanging drops, using the following conditions.

Glutamate mutase reconstituted with aquocobalamin was crystallized from 8% PEG 4000, 0.1 *M* Na acetate buffer pH 4.6 and 2 m*M* glutamic acid, maximum crystal size 0.5 × 0.4 × 0.04 mm. Glutamate mutase recombined with cyanocobalamin crystallized from 16% PEG 4000, 0.1 *M* tartrate, adjusted to pH 4.6 with NaOH, 2 m*M* glutamic acid, maximal crystal size 0.3 × 0.2 × 0.02 mm. Component S with cyanocobalamin crystallized from 28% PEG 4000, 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate, 6 m*M* KCl, maximum crystal size 0.15 × 0.15 × 0.02 mm.

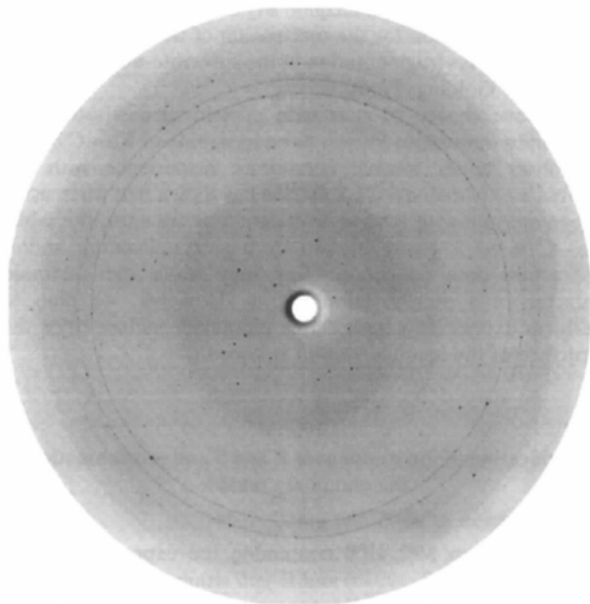

Fig. 1. Data collection (EMBL beamline X11, DESY, Hamburg) on component S with cyanocobalamin. Exposure time 4 min, oscillation range 1.0°.

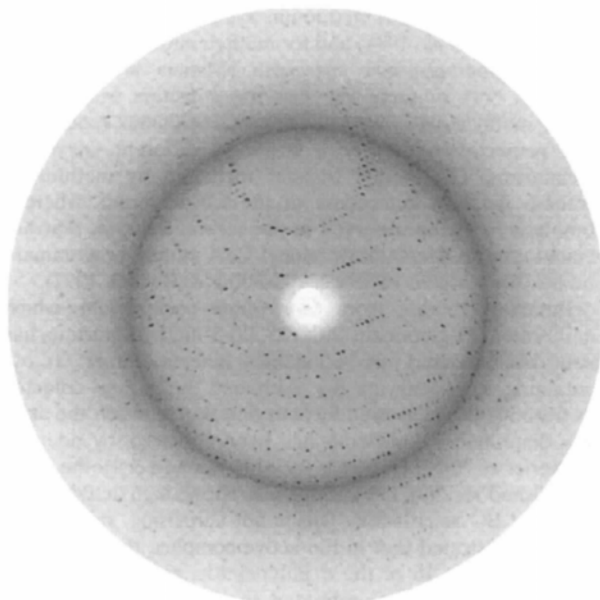

Fig. 2. Data collection (Siemens rotating anode, Cu $K\alpha$ radiation, graphite monochromator, MAR imaging plate) on glutamate mutase reconstituted with hydroxocobalamin. Exposure time 15 min, oscillation range 0.75°.

Table 1. *Data collection summary*

Protein	Space group	Cell dimensions (Å, °)	Data collected on	Resolution d_{min} (Å)	Completeness (%)	Completeness of the last shell (%)	$I/\sigma(I) > 3.0$ (%)	$I/\sigma(I)$ last shell > 3.0 (%)	R_{merge}†	Number of reflections
Component S with cyanocobalamin	$I422$	$a = b = 69.9$ $c = 107.1$	X11	3.2	30.00–3.20 Å 91.7	3.31–3.20 Å 99.6	81.4	85.5	0.12	82106 (total) 2395 (unique)
Glutamate mutase with aquo-cobalamin	$P2_1$	$a = 64.6$ $b = 113.2$ $c = 108.4$ $\beta = 96.0$	Rotating anode	2.8	20.00–2.8 Å 94.4	2.86–2.80 Å 97.3	69.1	48.3	0.11	264145 (total) 38311 (unique)
Glutamate mutase with cyano-cobalamin	$P2_1$	$a = 64.1$ $b = 112.8$ $c = 108.1$ $\beta = 95.7$	Rotating anode	3.4	20.00–3.4 Å 84.7	3.38–3.40 Å 86.0	59.0	42.4	0.12	146777 (total) 21208 (unique)

† $R_{merge} = \sum\sum_i (I - I_i)^2 / \sum\sum_i (I_i^2)$.

4. X-ray investigations

All diffraction experiments were carried out at about 103 K. Crystals were soaked in a solution obtained by mixing the respective reservoir solution with 20–25% glycerol for 10–30 s, picked up with a nylon loop and flash cooled by immersing in liquid nitrogen. Diffraction data were collected either on a rotating-anode source (Siemens generator, equipped with a MAR imaging-plate detector, generator settings 40 kV, 80 mA, Cu $K\alpha$ radiation, apparent focus size 0.3 × 0.3 mm, graphite monochromator) or on one of the EMBL beamlines (X11) in Hamburg (Mar345 imaging plate, prototype, wavelength λ = 0.9076 Å). Diffraction data were processed with the program *DENZO* (Otwinowski, 1993; Minor, 1993). The native data collected so far for the two proteins are summarized in Table 1. One frame from each of the data collections on component S and on inactive glutamate mutase (aquocobalamin) are shown in Figs. 1 and 2, respectively.

For component S (complexed with cyanocobalamin) the molecular mass is 16.2 kDa and the unit-cell volume is 523 292 Å^3. With the assumption of 16 molecules per unit cell the Matthews number (Matthews, 1968) is 2.02 Å^3 Da^{-1}, with an expected range of 1.8–3.5 Å^3 Da^{-1}. Therefore, in space group $I422$ (16 asymmetric units), one molecule is likely to be present per asymmetric unit. This is consistent with the self-rotation function (Fig. 3), which only shows the origin peak.

The unit-cell volume for the 137 kDa inactive glutamate mutase with aquocobalamin is 788 356 Å^3. With two molecules per unit cell, the Matthews coefficient is V_M = 2.88 Å^3 Da^{-1}, consistent with one molecule of $\varepsilon_2\sigma_2$ per asymmetric unit. The self-rotation function (Fig. 4) shows two non-crystallographic peaks (66.7% of the origin peak) within or very close to the mirror plane of Laue class $2/m$. These two peaks are 90° apart, and it is clear that one of them has to originate from a non-

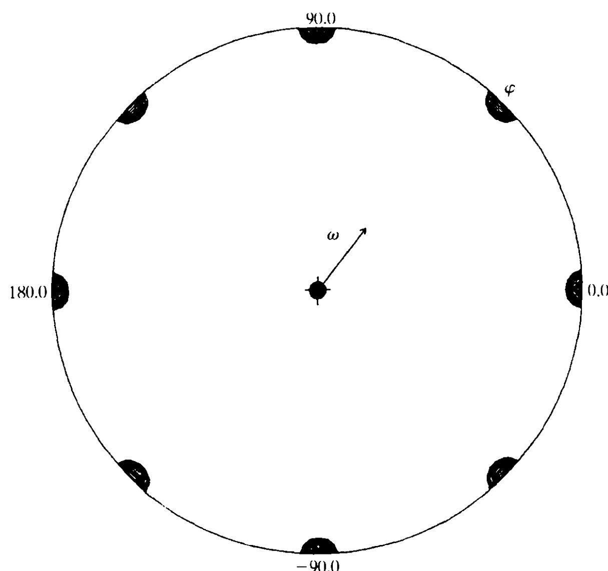

Fig. 3. Self-rotation function for component S with cyanocobalamin. A section at κ = 180° is shown. Data between a resolution of 10 and 4 Å were used, integration radius 25 Å. Contouring started at 15% in 5% increments. The axis conventions are as follows: κ is the rotation axis whose orientation with respect to the (orthogonalized) axes is defined by ω (angle from z axis) and φ (angle in the x, y plane).

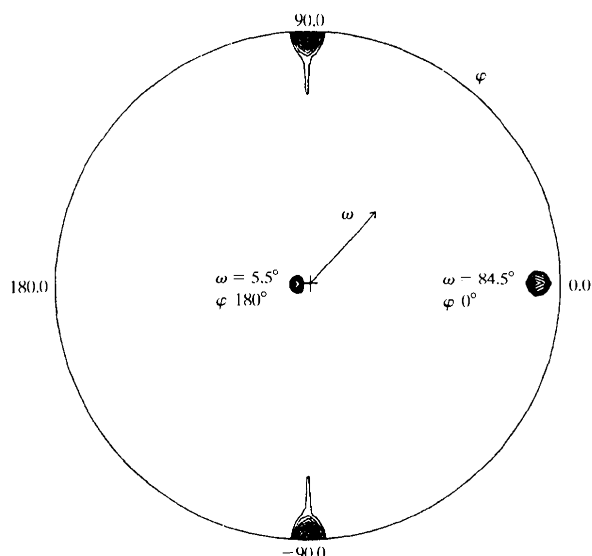

Fig. 4. Self-rotation function for glutamate mutase with aquocobalamin. Same settings as in Fig. 3, with the exception of the integration radius (50 Å). The angles (ω, φ) are given for the two non-crystallographic maxima.

crystallographic axis, probably relating one half of the $\varepsilon_2\sigma_2$ heterotetramer to the other half. Assuming one molecule of B_{12} per heterotetramer $\varepsilon_2\sigma_2$, the non-crystallographic dyad can obviously not hold for the B_{12} moiety. All the calculations for the self-rotation functions for component S and inactive glutamate mutase were performed with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

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