Cloning, purification, crystallization, and preliminary X-ray diffraction analysis of cystathionine γ-synthase from E. coli

ARTICLE *in* FEBS LETTERS · OCTOBER 1997

Impact Factor: 3.17 · DOI: 10.1016/S0014-5793(97)01057-0 · Source: PubMed

6 AUTHORS, INCLUDING:

Markus C Wahl
Freie Universität Berlin
120 PUBLICATIONS **5,594** CITATIONS
SEE PROFILE

Albrecht Messerschmidt
Max Planck Institute of Biochemistry
133 PUBLICATIONS **6,434** CITATIONS
SEE PROFILE

FEBS Letters 414 (1997) 492–496 FEBS 19205

Cloning, purification, crystallization, and preliminary X-ray diffraction analysis of cystathionine γ-synthase from *E. coli*

Markus C. Wahl, Robert Huber, Lars Prade, Snezan Marinkovic, Albrecht Messerschmidt, Tim Clausen*

Max-Planck-Institut für Biochemie, Abteilung Strukturforschung, Am Klopferspitz 18a, 82152 Planegg-Martinsried, Germany

Received 28 July 1997

Abstract The *Escherichia coli metB* gene has been PCR-extracted from genomic DNA and placed under the control of a *tac* and a T7 promoter in plasmids pCYB1 and pET22b(+), respectively, to produce overexpressing bacterial strains for the gene product, cystathionine γ-synthase. Efficient purification procedures have been developed for a C-terminally intein-tagged version and the wild-type target protein, yielding the product in a quantity and homogeneity amenable to high-resolution single-crystal X-ray analysis. Crystals have been obtained in space group P1 with unit cell constants a = 82.2 Å, b = 84.2 Å, c = 116.2 Å, α = 107.0°, β = 96.3°, γ = 108.0°, suggesting eight monomers per asymmetric unit (V_M = 2.23 Å^3/Da). Crystals diffract to beyond 2.6 Å resolution and a data set complete to 2.8 Å resolution has been collected using a rotating anode X-ray source. A cryogenic buffer system has been developed to allow synchrotron data collection. Patterson self rotation searches reveal the presence of two independent tetramers with local 222 symmetry in an asymmetric unit. The crystallographic results corroborate and extend previous solution studies regarding the quaternary organization of the enzyme.

Key words: Cystathionine γ-synthase; Methionine biosynthesis; Pyridoxal 5′-phosphate; X-ray crystallography; Protein crystallization; *Escherichia coli*

1. Introduction

De novo synthesis of methionine is carried out only by microorganisms and higher plants, suggesting that the enzymes involved are attractive targets for antibiotics and herbicide design. High resolution three-dimensional structures of the biological catalysts will prove invaluable in the quest for such agents. Cystathionine γ-synthase (CGS; EC 4.2.99.9; 386 residues, 40 kDa) catalyzes the committing step in methionine biosynthesis, which is the formation of L-cystathionine from *O*-succinyl-L-homoserine (OSHS) and L-cysteine in a pyridoxal 5′-phosphate (PLP) dependent γ-replacement reaction. *e*CGS is the product of the *metB* gene of *Escherichia coli* and belongs to the γ-family of PLP dependent enzymes [1]. It exhibits 30% sequence identity and 36% overall homology with *E. coli* cystathionine β-lyase (*e*CBL) [2], which catalyzes the subsequent step in the methionine pathway, the β-cleavage of L-cystathionine to homocysteine, pyruvate, and ammonia. This similarity indicates that CGS and CBL could have evolved from a common ancestor [2] and it is, not surprisingly, paralleled by a number of common structural and biochemical characteristics: both enzymes exert their functions in vivo as homo-tetramers with one PLP cofactor per monomer [3], which is bound via a Schiff base linkage to the ε-amino group of an active site lysine (K210 in *e*CBL, K198 in *e*CGS); CGS and CBL copurify in several systems [2,4]; and CGS can perform CBL-like β-eliminations, albeit with much reduced efficiency [5]. Therefore the overall tertiary structure and even the active site architecture of CGS should be closely related to that of CBL, whose X-ray structure has recently been solved and refined to 1.83 Å resolution [6].

Besides the above and the physiological reactions, CGS catalyzes in the absence of L-cysteine a γ-elimination from OSHS to succinate, α-ketobutyrate, and ammonia [3]. Moreover, it accomplishes β-replacement and α- or β-proton exchange reactions with appropriate substrates [3,7,8]. The acetylenic amino acid propargylglycine (2-amino-4-pentynoic acid) is a potent inactivator of plant and bacterial CGS [9–12] and can thus be regarded as a lead for the development of new inhibitors.

In this communication we present the design of *e*CGS overexpressing strains of *E. coli* and efficient purification protocols which yield over 80 mg of pure *e*CGS per liter of cell culture. Furthermore, the crystallization and diffraction data collection as well as the revealing results from Patterson self rotation analyses are described.

2. Materials and methods

2.1. Cloning and construction of expression strains

The *metB* gene [13] was amplified via PCR [14,15] with *E. coli* genomic DNA as the template. 5′-Overhangs introduced *Nde*I/*Sap*I (construct 1) and *Nde*I/*Bam*HI (construct 2) restriction sites into the forward/reverse primers (MWG-Biotech, Ebersberg, Germany; forward primer-1: 5′-GGA ATT CAT *ATG ACG CGT AAA CAG GCC ACC ATC G*-3′; reverse primer-1: 5′-AAT CAC GCT CTT CCG CAC *CCC TTG TTT GCA GCC CGG*-3′; forward primer-2: 5′-GCC GAA TTC CAT *ATG ACG CGT AAA CAG GCC ACC ATC*-3′; reverse primer-2: 5′-CGA CGG ATC CTA *TTA CCC CTT GTT TGC AGC CCG GAA GC*-3; restriction sites underlined, *e*CGS coding and template sequences in italics). The PCR products and vectors (construct 1: pCYB1, New England Biolabs, Beverly,

*Corresponding author. Fax: (49) (89) 8578-3516.
E-mail: clausen@biochem.mpg.de

Abbreviations: BSA, bovine serum albumin; CBL, cystathionine β-lyase; (*e*)CGS, cystathionine γ-synthase (*E. coli*); DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LB broth, Luria-Bertani broth; LB-Amp100, LB broth containing 100 μg/ml ampicillin; LDH, lactate dehydrogenase; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; NAD(H), nicotinamide adenine dinucleotide, oxidized (reduced) form; OSHS, *O*-succinyl-L-homoserine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEG, polyethylene glycol; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecylsulfate; Tris, tris-(hydroxymethyl)-aminomethane; V_M, Matthews coefficient (crystal volume per protein mass; Å^3/Da)

PII S0014-5793(97)01057-0

MA; construct 2: pET22b(+), Stratagene, La Jolla, CA) were digested with the appropriate restriction enzymes (New England Biolabs), ligated with T4 DNA ligase (New England Biolabs), and used for the electroporation (Gene Pulser, Bio-Rad, Hercules, CA) of *E. coli* strain XL1-Blue (Stratagene [16]). During cloning, all enzymatic steps were followed by heat inactivation of the enzymes and/or spin column purification of the desired DNA fragments (QIAquick, Qiagen, Chatsworth, CA). Individual colonies, selected on ampicillin (100 μg/ml) agar plates, were the source of plasmid preparations (Qiagen) for sequencing of the insert (Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Foster City, CA) and transformation of *E. coli* strains DH5α or BL21(DE3) for the pCYB1 and the pET22b(+) constructs, respectively.

2.2. Expression and purification of intein-tagged eCGS (preparation 1)

Individual colonies of the transformed *E. coli* strain DH5α (pCYB1 construct) were raised to inoculate 5 l of selective LB broth (100 μg/ml ampicillin; LB-Amp100). After growing at 37°C to an OD_{595} of 0.6–0.8, the cultures were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and allowed to grow for another 8 h. The cells were harvested by centrifugation (4000×*g*), resuspended in 10 ml buffer A (100 mM Tris-HCl, pH7.8, 10 μM PLP, 2 mM EDTA) per liter culture, and stored at −70°C. All subsequent procedures were carried out at 4°C or with the protein on ice. Chromatographic steps were conducted with self packed columns, peristaltic pumps, and gravity operated gradient mixers on Pharmacia Biotech (Uppsala, Sweden) UV detectors and fraction collectors.

The cell suspension was sonicated on ice (Branson Sonifier, Danbury, CT; macrotip, 100% output, 50% interval, 2×4 min) and the lysate cleared by ultracentrifugation (3 h, 50000 rpm, 55.2 Ti, Beckman Instruments, Palo Alto, CA). A 40 ml chitin column (New England Biolabs) was equilibrated, loaded, and washed as suggested by the manufacturer, except for the omission of detergents in the buffers, in order not to compromise the structural integrity and homogeneity of the tagged *e*CGS. Cleavage of the *e*CGS from the column bound tag and elution was achieved with a 30 mM DTT wash. The efflux was fractionated and checked on 12% polyacrylamide SDS gels for purity. Peak fractions were pooled and adjusted to 1 M $(NH_4)_2SO_4$ with a saturated, pH 7.5 buffered $(NH_4)_2SO_4$ solution. The protein was loaded onto a 75 ml phenyl Sepharose HP column (Pharmacia Biotech) and developed in a 500 ml gradient to 0 M $(NH_4)_2SO_4$. Fractions were again checked on gels, the *e*CGS peak pooled, concentrated and buffer exchanged to 10 mM Tris-HCl, pH 7.8, 10 μM PLP via Centriprep-10 concentrators (Amicon, Beverly, MA), and shock frozen in liquid nitrogen for storage at −70°C.

2.3. Expression and purification of wild-type eCGS (preparation 2)

In the case of the pET construct, the crude BL21(DE3) transformation mixture was used to inoculate a 50 ml LB-Amp100 culture, which was grown to log phase and aliquoted into glycerol stocks [17]. The glycerol stocks served to inoculate 6 l of LB-Amp100 which were subsequently grown, induced, harvested, stored, and lysed as above. All subsequent steps were performed at 4°C or with the protein on ice. The cleared lysate was loaded onto a 500 ml DEAE Sepharose FF column (Pharmacia Biotech), equilibrated with buffer A, and eluted with a linear gradient of 0–0.5 M $(NH_4)_2SO_4$ in buffer A. *e*CGS peak fractions were identified by low OD_{280}/OD_{426} absorbance ratios (DU 7500 spectrophotometer, Beckman Instruments) and conservatively pooled after checking their purity and molecular weight with 12% SDS PAGE. Pooled fractions were adjusted to 1 M $(NH_4)_2SO_4$ as above, chromatographed on a 75 ml phenyl Sepharose HP column (Pharmacia Biotech) equilibrated with buffer A plus 1 M $(NH_4)_2SO_4$, and eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in buffer A. The *e*CGS was identified on gels as before, concentrated in Centriprep-10 concentrators (Amicon), and further purified on a size exclusion column (Sephacryl S200 HR, Pharmacia Biotech) in 100 mM Tris-HCl, pH 7.8, 10 μM PLP. The final pool was adjusted to 60% $(NH_4)_2SO_4$ and the *e*CGS precipitate was stored at 4°C without activity loss throughout several months.

2.4. Activity test

*e*CGS activity was checked by the method of Kaplan and Flavin [18]. In a coupled assay, α-ketobutyrate, one product of the CGS catalyzed elimination reaction from OSHS, was reduced by lactate dehydrogenase (LDH) and NADH in a 1 ml solution (100 mM Tris-HCl, pH 7.8, 10 μM PLP, 0.3 mM NADH, 1.25 mM OSHS, 0.2 mg LDH from beef heart). Concomitant oxidation of NADH was spectroscopically monitored at 340 nm. *e*CGS concentrations were measured by a micro-BCA assay (Bio-Rad) against a BSA standard curve.

2.5. Crystallization and data collection

For crystallization trials *e*CGS was either thawed on ice (preparation 1) or its $(NH_4)_2SO_4$ pellet (preparation 2) harvested by centrifugation (25000 rpm, 20 min, JA-25.50, Beckman Instruments), resuspended in buffer A, and extensively dialyzed against 10 mM Tris-HCl, pH 7.8, 10 μM PLP. The protein concentration was adjusted to 15 mg/ml to search for crystallization conditions at room temperature and 4°C with a 96 condition sparse matrix screen. For both *e*CGS preparations the most promising crystals appeared at room temperature by vapor equilibration of sitting drops against 100 mM HEPES-NaOH, pH 7.0, 20% (w/v) PEG-4000, 10% (v/v) 2-propanol within several weeks. The preparation 1 crystals were improved by screening of additives. While additives were of no use for preparation 2 *e*CGS, these crystals were optimized via pH PEG-4000 grid screens. Data were collected at 113 K on a MAR Research (Hamburg, Germany) image plate detector equipped with a Rigaku (Tokyo, Japan) RU 200 rotating anode, producing graphite monochromated CuKα radiation (λ=1.5418 Å) at 50 kV/100 mA, and a liquid nitrogen cryosystem (KGV, Karlsruhe, Germany). The apparent focal spot size was 0.4 mm×0.4 mm. A single preparation 2 specimen sufficed to collect a 180° φ scan with a 1.0° frame width and 1800 s exposure time per frame. The data were processed with the program packages MOSFLM [19] and CCP4 [20] and self rotation functions were calculated with the program GLRF [21].

3. Results and discussion

3.1. Conventional and affinity tagged purification

*e*CGS could be expressed without complications to high levels in *E. coli* both with a C-terminal affinity tag (self cleavable intein domain plus chitin binding domain) and as the wild type protein under the control of a *tac* or a T7 promoter, respectively. The recently introduced intein/affinity tag (Impact I, New England Biolabs) represents a potential one-step purification tool for recombinant proteins [22]. The tagged version released untagged *e*CGS upon DTT induced

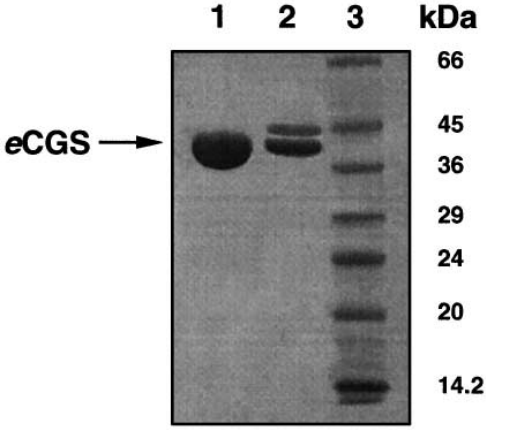

Fig. 1. 12% reducing SDS PAGE showing the products of the two *e*CGS preparations. Lane 1: *e*CGS preparation 2. Lane 2: *e*CGS preparation 1. Lane 3: Molecular weight markers (Sigma, Deisenhofen, Germany); low range marker, 66 kDa, BSA; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle); 29 kDa, carbonic anhydrase (bovine erythrocytes); 24 kDa, trypsinogen (bovine pancreas); 20 kDa, trypsin inhibitor (soybean); 14.2 kDa, α-lactalbumin (bovine milk).

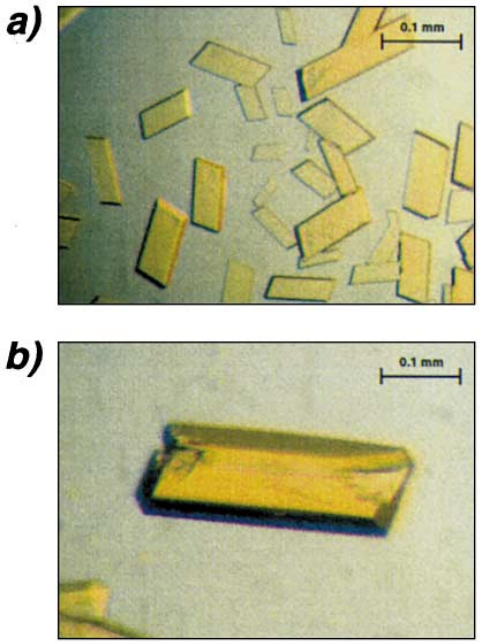

Fig. 2. Crystals of *e*CGS (preparation 2). Crystallization setup as described in the text. Note the influence of the pH. The two photographs are on the same scale. a: 100 mM MES/Tris, pH 7.5, 18% (w/v) PEG-4000, 10% (v/v) 2-propanol. b: 100 mM MES/Tris, pH 7.2, 18% (w/v) PEG-4000, 10% (v/v) 2-propanol.

on-column cleavage between the last residue of *e*CGS and the first residue of the intein/chitin binding portion. However, despite extensive washing steps, the preparation was not pure enough to be used in crystallization trials after the affinity column, necessitating a subsequent hydrophobic interaction chromatography step. The purity could probably be improved by incorporating detergents in the washing buffers. On SDS gels the product presented itself as a double band with a major (about 2/3) component running at the expected molecular weight (40 kDa), and a minor component with an apparent molecular weight of ~45 kDa (Fig. 1). The two bands comigrated in all attempted chromatographic procedures (chitin beads, phenyl Sepharose HP, Sephacryl S200 HR) and did not disappear upon prolonged boiling before loading of the gels. The excellent OD_{280}/OD_{426} ratio (3.5) of the preparation suggests that both components are *e*CGS, which might be inseparable under native conditions due to the formation of hetero-tetramers. N-terminal sequencing did not detect any heterogeneity leaving the possibility for extra residues on the C-terminus. The 6 l culture yielded about 30 mg of target protein.

In order to avoid the apparent heterogeneity, *e*CGS was purified by conventional means from a T7 controlled expression system. The three-column procedure could be conveniently completed within 3 days and the *e*CGS product advanced as a single band of the expected molecular weight on SDS gels (Fig. 1). Over 500 mg of pure, crystallizable protein with an OD_{280}/OD_{426} ratio of 3.3 has been prepared from 6 l of cell culture with enrichment factors comparable to previous reports [23].

Both *e*CGS preparations exhibited similar specific activities when tested in a continuous coupled assay [18], further suggesting that the double bands of preparation 1 constitute the same enzyme. Both products were extremely stable and did not show any significant activity loss when stored in various environments at room temperature, 4°C, or deep frozen.

3.2. Crystallization, data collection, and self rotation

Both *e*CGS preparations yielded crystals under the same conditions in the preliminary trials, using sitting drop vapor diffusion with 4.5 μl drops (3 μl protein at 15 mg/ml plus 1.5 μl reservoir) against a 500 μl reservoir (100 mM HEPES-NaOH, pH 7.0, 20% (w/v) PEG-4000, 10% (v/v) 2-propanol). The preparation 1 crystals could be improved through trace amounts of benzamidine to yield voluminous needles (0.05 Å × 0.05 Å × 1.0 Å) which diffracted to about 3.5 Å resolution. The initial preparation 2 crystals were larger in all dimensions by a factor of 2 and were selected for further refinement. Addition of additives did not have visible effects. Fine screening of the PEG-4000/pH combinations, replacing 2-propanol with MPD, and using hanging drops yielded the best crystals so far, which grew in the pH range of 7.0–7.5 and at PEG-4000 concentrations above 17%. MPD instead of 2-propanol avoided the formation of additional spherolytic crystals and convection currents which were an obstacle in mounting. The crystal size and shape was very sensitive to the pH with lower values producing a larger number of small, perfectly shaped crystals (Fig. 2a) and higher values promoting the formation of fewer, larger crystals with some growth defects at the edges (Fig. 2b).

Preliminary data collection was attempted with the crystals mounted in thin-walled capillaries with mother liquor at one end. However, the low symmetry space group (P1) required data collection over several days, necessitating the search for cryogenic buffers. It was found essential to keep the pH in these buffers exactly the same as in the reservoir, and pH 7.2–

Table 1
Crystallographic data

Space group	P1 (triclinic)	
Unit cell dimensions	a = 82.2 Å, b = 84.2 Å, c = 116.2 Å	α = 107.0°, β = 96.3°, γ = 108.0°
Resolution range	∞–2.8 Å	
Reflections	Observed	128 009
	Unique	65 350
	$F \geq 3\sigma(F)$	55 060
	% Completeness (10.0–2.8 Å) all data/$F \geq 3\sigma(F)$	96%/83%
	% Completeness (2.9–2.8 Å) all data/$F \geq 3\sigma(F)$	95%/58%
	Redundancy	2.0
R_{merge}	10.0–2.8 Å	11.7%
	2.9–2.8 Å	39.3%
Asymmetric unit	8 *e*CGS monomers	
V_M	2.23 $Å^3$/Da	

Table 2
Self rotation function[a] peaks

Peak number	Φ	Ψ	σ-Level
1	100	72	8.1
2	94	74	6.6
3	114	164	6.5
4	6	96	6.3
5	16	92	4.6
6	110	156	4.4
7	162	146	2.8

[a]κ = 180°; polar convention XZK, orthogonalization AXABZ according to GLRF [21].

7.3 crystals clearly survived the shock freezing best. Otherwise it sufficed to raise the MPD concentration to about 30%. Similar buffer systems should be useful for the collection of synchrotron radiation data. With a rotating anode source, the crystals diffracted to beyond 2.6 Å resolution and yielded a complete data set to 2.8 Å (Table 1). The space group was determined to be triclinic P1 (a = 82.2 Å, b = 84.2 Å, c = 116.2 Å; α = 107.0°, β = 96.3°, γ = 108.0°), suggesting eight *e*CGS monomers per asymmetric unit (V_M = 2.23 Å^3/Da, solvent content 32%). The R_{merge} was 11.7% for 128 009 reflections, measured with a 2-fold redundancy, of which 55 060 were unique above the 3σ level (in F) and were used in the preliminary crystallographic characterization (Table 1).

Locked Patterson self rotation functions [21] were calculated with κ = 90° and κ = 180° (Table 2), screening the asymmetric unit in 2° intervals. The results clearly indicated the presence of two groups of three local orthogonal twofold axes (Fig. 3), implying the presence of two *e*CGS tetramers with local 222 symmetry slightly inclined with respect to each other.

3.3. Biological significance

Although the purification of native and recombinant CGS from prokaryotic and eukaryotic organisms has been demonstrated previously [7,12,23,24], we have tailored the preparation for the first time to the needs of X-ray crystallography, demonstrating the reproducible generation of high quality single crystals. In terms of product yield and purity as well as with respect to its convenience the presented procedures match or improve upon previous protocols.

The Patterson analysis of the current diffraction data clearly supports the solution studies indicating that *e*CGS is organized in tetramers. Furthermore, these tetramers should exhibit 222 symmetry. Although *e*CGS is therefore similarly structured as *e*CBL on the quaternary level, and although the enzymes share significant sequence homology, the structure solution by molecular replacement of the present crystals, with eight monomers per asymmetric unit, did not prove to be trivial. Currently structure solution strategies via molecular and isomorphous replacement are attempted. At the same time we are pursuing cocrystallization with inhibitors.

CGS activities cover a large portion of the spectrum of known PLP dependent reactions, with eliminations and/or substitutions at the α, β, and γ positions of substrates [3]. At the same time CGS is the only enzyme which sponsors a physiological substitution at C^{γ} of an amino acid. It is therefore well suited as a subject to study the mechanisms by which substrates are funnelled into either route. Mechanistic details

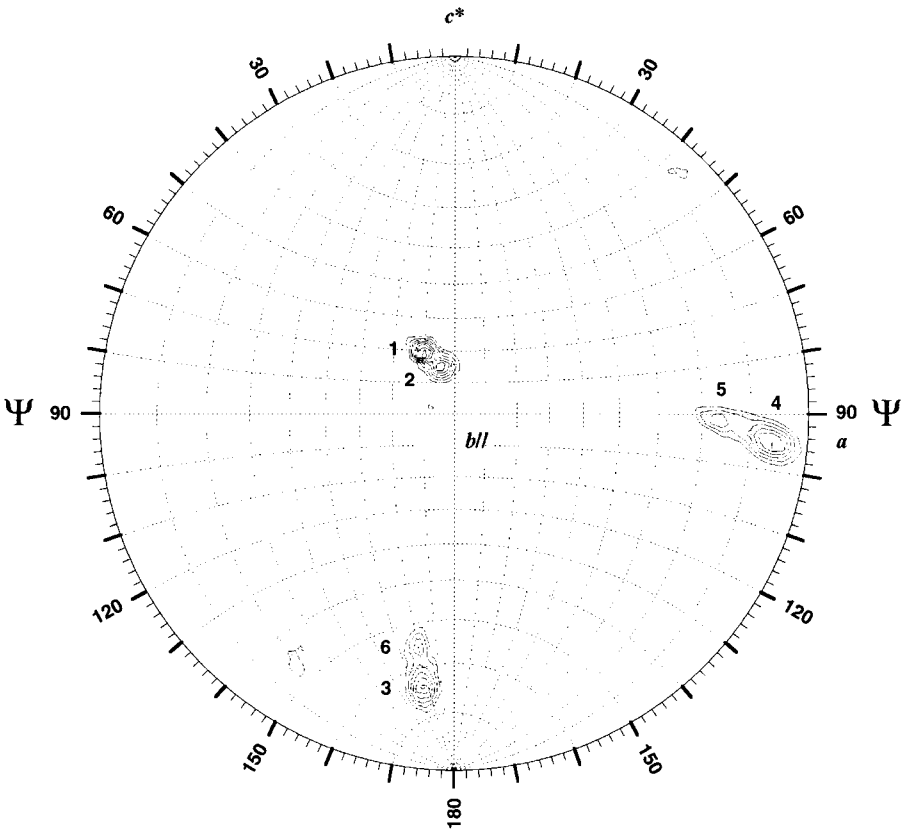

Fig. 3. Results from a locked self rotation function contoured with a cutoff of 2.5σ. The peaks indicate two groups of three orthogonal axes (Table 2).

for the reaction mechanism of CGS have been proposed [24–26] and recently modified by stopped-flow kinetic measurements [3]. It is hoped that the three-dimensional structure will improve our understanding of CGS catalyzed reactions. So far the structure of only one member of the structural γ-family of PLP dependent enzymes, CBL from *E. coli* [6], has been determined. While there exists extensive homology between the two enzymes they catalyze vastly different reactions in vivo. It will be interesting to see how the differential active site architectures direct the different chemical scenarios.

Acknowledgements: Generous financial support from the Peter und Traudl Engelhorn Stiftung zur Förderung der Biotechnologie und Gentechnik to M.C.W. is gratefully acknowledged.

References

[1] Alexander, F.W., Sandmeier, E., Mehta, P.K. and Christen, P. (1994) Eur. J. Biochem. 219, 953–960.
[2] Belfaiza, J., Parsot, C., Martel, A., Bouthier de la Tour, C., Margerita, D., Cohen, G.N. and Saint-Girons, I. (1986) Proc. Natl. Acad. Sci. USA 83, 867–871.
[3] Brzovic, P., Litzenberger Holbrook, E., Greene, R.C. and Dunn, M.F. (1990) Biochemistry 29, 442–451.
[4] Guggenheim, S. (1971) Methods Enzymol. 17, 439–442.
[5] Flavin, M. (1975) in: Metabolic pathways (Greenberg, D.M., Ed.) vol. 7, pp. 457–503, Academic Press, New York.
[6] Clausen, T., Huber, R., Laber, B., Pohlenz, H.-D. and Messerschmidt, A. (1996) J. Mol. Biol. 262, 202–224.
[7] Guggenheim, S. and Flavin, M. (1969) J. Biol. Chem. 244, 3722–3727.
[8] Guggenheim, S. and Flavin, M. (1969) J. Biol. Chem. 244, 6217–6227.
[9] Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K. and Walsh, C. (1979) Biochemistry 18, 4691–4701.
[10] Datko, A.H. and Mudd, S.H. (1982) Plant Physiol. 69, 1070–1076.
[11] Thompson, G.A., Datko, A.H. and Mudd, S.H. (1982) Plant Physiol. 70, 1347–1352.
[12] Kreft, B.D., Townsend, A., Pohlenz, H.D. and Laber, B. (1994) Plant Physiol. 104, 1215–1220.
[13] Zakin, M.M., Greene, R.C., Dautry-Varsat, A., Duchange, N., Ferrara, P., Py, M.-C., Margarita, D. and Cohen, G.N. (1982) Mol. Gen. Genet. 187, 101–106.
[14] Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Science 230, 1350–1354.
[15] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487–491.
[16] Smith, M., Jessee, J., Landers, T. and Jordan, J. (1991) Focus 12, 38–40.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989), Molecular Cloning: A Laboratory Manual (Nolan, C., Ed.) Vol. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Kaplan, M. and Flavin, M. (1966) J. Biol. Chem. 241, 5781–5789.
[19] Leslie, A.G.W. (1990) MOSFLM program. Abstract of the Crystallographic Computing School, pp. 27–38, Bischenberg.
[20] Collaborative Computational Project Number 4 (1994) Acta Crystallogr. D50, 760–763.
[21] Tong, L. and Rossman, M.G. (1990) Acta Crystallogr. A46, 783–792.
[22] Chong, S., Yang, S., Paulus, H., Brenner, J., Perler, F.B. and Xu, M.-Q. (1996) J. Biol. Chem. 271, 22159–22168.
[23] Litzenberger Holbrook, E., Greene, R.C. and Heilig Krueger, J. (1990) Biochemistry 29, 435–442.
[24] Johnston, M., Marcotte, P., Donovan, J. and Walsh, C. (1979) Biochemistry 18, 1729–1738.
[25] Davis, L. and Metzler, D.E. (1972) Enzymes 7, 33–74.
[26] Walsh, C.T. (1979) Enzymatic Reaction Mechanisms, pp. 777–827, Freeman, San Francisco, CA.