Crystal structure analysis of a pentameric fungal and an icosahedral plant lumazine synthase reveals the structural basis for differences in assembly

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Abstract

Lumazine synthase catalyzes the penultimate step in the synthesis of riboflavin in plants, fungi, and microorganisms. The enzyme displays two quaternary structures, the pentameric forms in yeast and fungi and the 60-meric icosahedral capsids in plants and bacteria. To elucidate the structural features that might be responsible for differences in assembly, we have determined the crystal structures of lumazine synthase, complexed with the inhibitor 5-nitroso-6-ribitylamino-2,4-pyrimidinedione, from spinach and the fungus *Magnaporthe grisea* to 3.3 and 3.1 Å resolution, respectively. The overall structure of the subunit and the mode of inhibitor binding are very similar in these enzyme species. The core of the subunit consists of a four-stranded parallel β -sheet sandwiched between two helices on one side and three helices on the other. The packing of the five subunits in the pentameric *M. grisea* lumazine synthase is very similar to the packing in the pentameric substructures in the icosahedral capsid of the plant enzyme. Two structural features can be correlated to the differences in assembly. In the plant enzyme, the N-terminal β -strand interacts with the β -sheet of the adjacent subunit, thus extending the sheet from four to five strands. In fungal lumazine synthase, an insertion of two residues after strand β 1 results in a completely different orientation of this part of the polypeptide chain and this conformational difference prevents proper packing of the subunits at the trimer interface in the icosahedron. In the spinach enzyme, the β -hairpin connecting helices α 4 and α 5 participates in the packing at the trimer interface of the icosahedron. Another insertion of two residues at this position of the polypeptide chain in the fungal enzyme disrupts the hydrogen bonding in the hairpin, and the resulting change in conformation of this loop also interferes with proper intrasubunit contacts at the trimer interface.

Keywords: inhibitor; macromolecular assembly; protein crystallography; riboflavin synthesis; substrate analogue

Riboflavin, vitamin B₂, is the precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide, cofactors that are essential for the activity of a wide variety of metabolic enzymes involved in electron transfer. Riboflavin is produced by plants, fungi, and microorganisms while mammals must acquire it through their diet (for recent reviews see Bacher, 1990; Bacher et al., 1996, 1997). The penultimate step in the biosynthetic pathway of riboflavin (Fig. 1) is mediated by lumazine synthase (LS) that catalyzes the condensation of 5-amino-6-(D-ribitylamino)-2,4(1H,3H)-pyrimidinedione (ARAPD) with 3,4-dihydroxy-2-butanone-4-phos-

phate (DHBP) yielding one molecule each of 6,7-dimethyl-8-ribityllumazine (DMRL) and orthophosphate (Kis et al., 1995). In the subsequent step, two molecules of DMRL dismutate yielding riboflavin and ARAPD. This part of the reaction is catalyzed by riboflavin synthase (RS) and has been studied extensively (Plaut et al., 1970; Plaut & Harvey, 1971; Bacher, 1990). ARAPD, formed in the second half of the reaction, is reutilized as substrate for lumazine synthase; thus, every second molecule of pyrimidine passes twice through the catalytic cycle.

The enzymes catalyzing the last two steps in riboflavin synthesis, LS and RS, are the best characterized of this biosynthetic pathway (Bacher, 1990; Bacher et al., 1996, 1997). In *Bacillus subtilis* the two enzymes form a 1 MDa complex composed of three subunits of RS (α), and 60 subunits of LS (β). The 60

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β -subunits pack with 532 symmetry forming an icosahedral capsid that encloses an α -trimer in its central core (Bacher et al., 1980). It has been suggested that the tight packing arrangement of LS and RS improves the catalytic efficiency by substrate channeling at low substrate concentrations (Kis & Bacher, 1995). The $\alpha_3\beta_{60}$ complex as well as the hollow β_{60} complex from *B. subtilis* have previously been studied by X-ray crystallography (Ladenstein et al., 1988, 1994; Ritsert et al., 1995). In *Escherichia coli*, LS exists as hollow β_{60} capsids. Plant lumazine synthases are synthesized as precursor proteins with an N-terminal chloroplast targeting sequence. After post-translational import into the chloroplast stroma, the signal peptide is cleaved off yielding the mature protein that forms icosahedral capsids (D. Jordan & P. Viitanen, unpubl. results).

The enzymes from fungi, such as *Saccharomyces cerevisiae* (Mörtl et al., 1996) and *Magnaporthe grisea*, are pentamers and thus display a different quaternary structure. The fungus *M. grisea* is a rice parasite that causes severe losses in rice production worldwide. As LS is a potential target for the design of antifungal agents for protecting crop plants, it is of interest to compare the fungal enzyme to its plant counterpart so that selective inhibitors may be devised. The lumazine synthase from *M. grisea* (MLS) as well as mature spinach lumazine synthase (SLS) have been expressed in *E. coli*, purified to homogeneity and crystallized as a complex with a substrate analogue, 5-nitroso-6-ribitylamino-2,4-pyrimidinedione (Fig. 1B). Here we present the crystal structures of the icosahedral spinach LS, determined at 3.3 Å and the pentameric LS from *M. grisea* determined to 3.1 Å resolution. The structures of the core of the monomers are very similar, despite their different packing arrangements. Comparison of icosahedral and pentameric lumazine synthase provides insights into the structural basis for differences in assembly.

Fig. 1. A: The reaction catalyzed by lumazine synthase. **B:** Molecular structure of the inhibitor 5-nitroso-6-ribitylamino-2,4-pyrimidinedione, used in these studies.

Table 1. Data collection and refinement statistics

	Spinach	<i>M. grisea</i>
Data collection		
Resolution (Å)	25–3.3	20–3.1
Highest resolution shell	3.36–3.30	3.17–3.10
Measured reflections	801,553	539,917
No. of unique reflections	52,248	31,842
Completeness (%)	98.3 (98.3)	99.1 (91.5)
<i>R</i> _{merge} (%)	11.1 (33.8)	13.6 (39.6)
<i>I</i> / σ (<i>I</i>)	12.1 (3.9)	12.0 (3.3)
Refinement		
Resolution (Å)	12–3.3	20–3.1
Number of unique reflections	50,119	31,795
<i>R</i> _{factor} (%)	32.0 (31.0)	24.8
<i>R</i> _{free} (%)	33.0 (32.6)	27.1
Number of protein atoms	1,163*20	1,245*10
Average <i>B</i> -factor	44.7	23.3
RMS bond length deviation (Å)	0.014	0.009
RMS bond angle deviation (deg)	1.713	1.219
Ramachandran statistics (%)		
Most favored regions	81.5	95.6
Additional allowed regions	15.6	3.6
Generously allowed	2.2	0.0
Disallowed	0.7	0.7

Results

Electron density maps and structure quality

The crystal structure of SLS was determined by molecular replacement at 3.3 Å resolution and has been refined to an *R*-value of 31.0% (*R*_{free} = 32.6%) with good stereochemistry (Table 1). The quality of the final 20-fold averaged 3.3 Å electron density map is generally good. The polypeptide chain is well defined except for two surface loops, comprising residues 42–44 and 84–87, with weak electron density and a few flexible side chains located on the surface of the protein. The C-terminal residue Ala156 is not visible in the electron density map, probably due to disorder, and has therefore been excluded from the final model. In the final, refined electron density map, the substrate analogue is well defined.

The structure of MLS, at 3.1 Å resolution, was also solved using molecular replacement. The final 10-fold averaged map is of very good quality except for two regions for which no electron density could be seen; the first four residues at the N-terminal and a segment of 36 residues. This long loop, unique for MLS, is located on the surface of the molecule and is not involved in substrate binding or crystal packing. Otherwise, most side chains are clearly visible in the electron density map, except for some of those located in the turn separating the final two helices where only the main chain was built. In the final model, comprising two pentamers, each subunit contains 165 residues, one inhibitor molecule, and a sulfate ion in a putative second substrate binding cleft. In 7 of the 10 subunits of the asymmetric unit, a second sulfate ion, far from the active site, is included in the model. In addition to these 17 sulfates, one sulfate ion and five water molecules have been modeled in each of the pores that are formed around the fivefold axis of the pentamer. Due to the low resolution of the structure analysis, the assignment of solvent at these positions, however,

Fig. 2. **A:** Superposition of the subunits of lumazine synthase from spinach (blue) and *M. grisea* (red). **B:** Comparison between the pentamers in lumazine synthase from spinach (left) and *M. grisea* (right). The subunits are shown in different colors. The N-terminal strands are highlighted in red to illustrate the different conformation in the two enzymes.

must be considered tentative. The structure of MLS has been refined to an *R*-value of 24.8% ($R_{\text{free}} = 27.1\%$). Details of the refinement and model statistics are given in Table 1.

Spinach lumazine synthase

Overall fold of the monomer

The SLS monomer comprises 156 amino acids and forms a single domain (Fig. 2A) with an overall fold similar to that of bacterial LS (Ladenstein et al., 1988). The secondary structure elements as defined by the program DSSP (Kabsch & Sander, 1983) together with an alignment of the available amino acid sequences for lumazine synthase are displayed in Figure 3. The core of LS is a repetition of four β/α motifs and is characterized by a parallel β -sheet with helices on both sides. The central four-stranded β -sheet has $\beta 3\beta 2\beta 4\beta 5$ topology. The N-terminal residues fold into strand $\beta 1$ that protrudes from the core of the monomer forming the fifth parallel strand in the β -sheet of an adjacent subunit. This pattern is repeated within five subunits resulting in a pentameric substructure (Fig. 2B). On one side of the central β -sheet,

the two helices $\alpha 2$ and $\alpha 3$ are running in parallel and three helices are located on the other side of the β -sheet, with $\alpha 1$ and $\alpha 4$ almost parallel to each other. The last two helices, $\alpha 4$ and $\alpha 5$, are separated by a short β -hairpin (residues 128–134) at an angle of 50° . This structural feature is stabilized by a number of hydrogen bonds.⁵ Two main-chain hydrogen bonds stabilize the β -hairpin structure. Another hydrogen bond is formed between the carbonyl oxygen of Arg127, which is the last residue of $\alpha 4$, and the amide group of Lys135, the first residue of helix $\alpha 5$. In addition to this helix-capping interaction, the side-chain oxygen of the conserved residue Asn134 participates in two hydrogen bonds with the NH groups of residues 136 and 137. In this way, the conformation of the $\alpha 4$ -hairpin- $\alpha 5$ motif is well defined, and the significance of this motif will be discussed below.

⁵The present resolution of the electron density maps does not allow the unambiguous assignment of hydrogen bonds. In the following, this term is used simply to identify those atoms in the refined models of MLS and SLS atoms, which can act as hydrogen-bond donors or acceptors and which are within a distance usually found for such bonds, 2.7–3.2 Å.

Fig. 3. Multiple sequence alignment of lumazine synthases. Secondary structure elements are shown above the sequences: hhh, α -helices; eee, β -strands found both in spinach and in fungal LS; bbb, β -strands found only in the spinach enzyme. Residues of the spinach enzyme participating in pentamer contacts are indicated by *, those participating in trimer contacts by #. Residues involved in binding of the inhibitor and the sulfate ion at the active site are denoted by #. The numbering of amino acids is that of lumazine synthase from *B. subtilis*. Sequences from the following organisms were used: *Eremothecium gossypii* (g2300730), *S. cerevisiae* (RIB4_YEAST), *M. grisea*, *Dehalosporium multivorans* (g3002553), *Helicobacter pylori* (g2313079), *Bacillus amyloliqueficans* (RISB_BACAM), *B. subtilis* (RISB_BACSU), *Actinobacillus pleuropneumoniae* (RISB_ACTPL), *E. coli* (RISB_ECOLI), *Haemophilus influenzae* (RISB_HAEIN), *Photobacterium leiognathi* (RISB_PHOLE), *Photobacterium phosphoreum* (RIBH_PHOPO), *Synechocystis* sp. (g1652647), *Arabidopsis thaliana*, *Nicotiana tabacum*, *Spinacia oleracea*, *Chlamydia trachomatis* (g3329190), *M. tuberculosis* (RISB_MYCTU), *B. abortus* (g2304197), *M. jannaschii* (RISB_METJA).

All four strands of the central sheet are connected to the following α -helices by either β -turns or short loops, the longest loop between $\beta 4$ and $\alpha 3$ contains seven residues. All these loops or turns are rather conserved. Residues from three of the four connections participate in the binding of the substrate analogue (see below). The loops connecting the α -helices with the following β -strands are also rather short; the longest and least conserved loop between $\alpha 1$ and $\beta 3$ consists of six residues (Fig. 3). Thus, the subunit of SLS can be described as a one-domain compact globule without large flexible parts extending into the solvent.

The icosahedral capsid

Sixty monomers of SLS assemble into an icosahedral capsid with a diameter of about 160 Å (Fig. 4). The capsids formed by recombinant mature spinach LS are hollow; the diameter of the internal cavity is 70–75 Å, with a volume of $\sim 2 \times 10^5$ Å³. This volume would be sufficient to encapsulate a trimer of RS, assuming that plant RS has a similar size as its bacterial counterpart.

The icosahedron has a triangulation number $T = 1$, which implies that all participating building blocks occupy identical posi-

tions. The capsid can be described as a dodecamer of pentamers with all subunits forming pentameric, trimeric, and dimeric contacts. Every monomer is arranged in the capsid in such a way that all loops connecting β -strands with the following helices, i.e., the active sites, are located in the inner part of the capsid. The direction of strand $\beta 5$ roughly coincides (the deviation is about 20°) with the direction to the center of the capsid. This does not hold for all strands due to a twist in the sheet (Fig. 2A). The N- and C-termini of the monomers are located outside the capsid.

The pentamer is the most pronounced substructure and forms the most extensive interactions. Upon formation of pentamers, 2,550 Å² of each subunit surface is buried, corresponding to 32.5% of the total solvent accessible area of the monomer. The interface includes strand $\beta 1$ of one subunit that interacts with strand $\beta 3$ of an adjacent subunit, interactions between helices $\alpha 3$ from two subunits, and helix $\alpha 5$ of one subunit with helices $\alpha 3$ and $\alpha 1$ of the other. Residues involved in intersubunit contacts at this interface are indicated in Figure 3. In addition to hydrogen bonds, there are several salt bridges in the interface region, one salt bridge involves three residues, Glu5, Arg22, and Glu145.

Fig. 4. View of the icosahedral capsid of spinach lumazine synthase. The pentamers are shown in different colors.

The fivefold axis is surrounded by helices $\alpha 3$ from the five subunits forming a channel, 11–12 Å wide in SLS (Fig. 2B). This channel is the largest opening in the SLS capsid and the most probable pore for transportation of substrates and products to and from the interior of the capsid where the active sites of the β -subunits, as well as the riboflavin synthase trimers in the case of *B. subtilis*, are found. The size of the BLS pore is not sufficient to permit free transport of lumazine and riboflavin (Ladenstein et al., 1988). This is not the case for SLS where the channel is large enough to allow for passage of the substrates, products, and also for riboflavin. Figure 5 shows riboflavin manually docked inside the pore. The analysis of the close contacts between docked riboflavin and protein atoms, using the program CONTACTS (CCP4, 1994), showed that a rigid model of riboflavin can be fitted into the pore without sterical clashes.

The dimeric contacts between subunits are rather few and are located on the outer part of the capsid. This interface covers 400 Å² of each of the participating monomers. The trimeric contacts are mainly formed by three segments of the polypeptide chain comprising residues 22–30, residues 37 and 82–84, and finally residues 121–131. This interface covers ~900 Å² or 11% of each subunit in the trimer. All residues closer than 3.5 Å and involved in packing interactions across the threefold axis are indicated in Figure 3. Residues from the hairpin between helices $\alpha 4$ and $\alpha 5$ are very close to the N-terminal end of $\alpha 1$. The conformation of Gly129 ($\phi, \psi = 91^\circ, 22^\circ$) places the main-chain oxygen O129 in a position to form a hydrogen bond with NH of residue 23 from the adjacent subunit in the trimer. The trimer contact surface has the shape of a funnel with its mouth on the inner wall of the capsid. This side of the funnel is negatively charged as Asp122 from each of the three participating subunits is located at the inner surface close to the threefold axis. The other side of the funnel, at the outer surface, is positively charged due to the presence of Arg30 in the three

Fig. 5. The fivefold pore of spinach lumazine synthase. Residues of the $\alpha 3$ helices from the five subunits (in different colors) are shown as ball and stick models. A space-filling model of riboflavin is modeled into the pore to illustrate that the pore is large enough to allow for free diffusion of substrate and products. The picture was generated with MOLSCRIPT (Kraulis, 1991).

subunits. These layers of opposite charge are separated from each other by a hydrophobic layer, consisting mainly of residues Phe26 and Ile125.

The pyrimidine binding cleft

Lumazine synthase was crystallized with an analogue of the first substrate, 5-nitroso-6-ribitylamino-2,4-pyrimidinedione. The analogue is bound at the inner side of the capsid, on the interface of two neighboring subunits of the pentameric substructure (Figs. 2B, 6). Residues from three loops, connecting β -strands 2, 3, and 4 to the following helices, are participating in the binding of the inhibitor. The pyrimidine ring of the inhibitor is stacked with the side chain of Phe23 (distance ~3.4 Å) in a hydrophobic pocket formed by Phe23, Ala56, Val81, and Val92. The two keto groups of the pyrimidine ring interact with the side chain of Asn24 and the main-chain NH group of Ala56. The ribityl moiety is bound in a more hydrophilic region, facing strand $\beta 5$ of the neighboring subunit. The NH and CO groups of Phe113 form hydrogen bonds with two hydroxyl groups of the inhibitor, O11 and O12, respectively. Phe113 is the central residue of the very conserved strand $\beta 5$, and it is the only residue from the adjacent subunit that is directly involved in the binding of the inhibitor. In addition to Phe113, atoms of Val112 and Gly114 are at van der Waals distance from the inhibitor. The hydroxyl groups O9, O10, O11, and O12 of the ribityl tail form hydrogen bonds with Tyr57 (N), Glu58 (OD1), Phe113 (O), and with Glu58 (OD2), and Phe113 (N), respectively (Fig. 6). Additional hydrogen bonds might be formed via solvent molecules but cannot be detected at the present resolution of the electron density map. In the structure of BLS, a serine at position 142 forms a hydrogen bond, via a water molecule, to the O11 of the ribityl chain. This residue is necessary for catalytic activity

Fig. 6. Stereoview of the substrate binding cleft of spinach lumazine synthase. An annealed omit $F_o - F_c$ map, contoured at 2.5σ , is superimposed on the final refined model. The picture was generated by the program O (Jones & Kjeldgaard, 1997).

(Bacher et al., 1996). In SLS, this serine is conservatively exchanged for a threonine that might have the same importance for binding the substrate.

*Lumazine synthase from *M. grisea**

Structure of the subunit

The overall structure of the subunit of MLS (Fig. 2A) is generally very similar to BLS and SLS, despite the fact that MLS is pentameric while the other two enzymes form larger assemblies. The root-mean-square deviations (RMSDs) between 141 equivalent C α atoms are 0.99, 0.98, and 1.2 Å for BLS-SLS, BLS-MLS, and SLS-MLS superpositions, respectively. The polypeptide chain of MLS is 44 residues longer than the chain of the spinach enzyme. Superposition of the three-dimensional structures of SLS and MLS (Fig. 2A) shows that MLS has three insertions; most of the additional residues form a 36 amino acid long region between helix $\alpha 2$ and $\beta 4$. An insertion at this position is also present in the yeast enzyme although only five residues long (Mörtl et al., 1996). This insertion is of unknown function, and unfortunately it appears to be disordered in the crystal as no electron density was observed for

this region. These residues are present in the recombinant enzyme and not proteolytically removed, since electrospray MS experiments using purified MLS samples show the expected mass for the full-length enzyme. The region is very rich with hydroxyl-containing residues; there are ten serine and eight threonine residues between $\alpha 2$ and $\beta 4$ in MLS. This polypeptide segment might be of unique importance to the functionality of the fungal enzyme, and experiments are underway to test this hypothesis using truncated mutants.

In addition to this long stretch of 36 amino acids, there are two other, shorter insertions in MLS. Two additional residues are found just after strand $\beta 1$, and another two-residue insertion is located in the hairpin between helix $\alpha 4$ and $\alpha 5$. Both these short insertions are of significant consequence for the quaternary structure of MLS (see below). The first insertion, Pro8–Gln9 (Fig. 3), results in the different orientation of strand $\beta 1$ in MLS with respect to both the bacterial and plant enzyme; the N-terminal strand $\beta 1$ turns by 70°. Residues 5–11 are in an extended (β -) conformation like in SLS; however, they do not form the fifth strand of the sheet in the neighboring subunit, as they do in SLS or BLS. Instead, this chain segment points toward the turn between $\beta 2$ and $\alpha 1$ of the neighboring subunit that participates in the trimer interface (Fig. 7), and Gly5–Pro6 are very close to Trp25 of this subunit. In MLS the

Fig. 7. Modeled positions of the MLS subunits at the trimer interface of an icosahedron illustrating the disruption of the trimeric contacts in the fungal enzyme. The N-terminal segments from adjacent subunits in the pentamers, which disrupt the trimer interface, are shown in red. The picture was generated by MOLSCRIPT (Kraulis, 1991) and rendered by Raster3D (Merrit & Murphy, 1994).

Fig. 8. Stereoview of the substrate binding cleft of *M. grisea* lumazine synthase. A simulated annealing omit $F_o - F_c$ map, contoured at 2.5σ , is superimposed the final model. The picture was generated by the program O (Jones & Kjeldgaard, 1997).

chain segment comprising residues 5–11 is thus not part of any β -sheet, for sake of simplicity it is nevertheless denoted strand β 1 in the following comparisons. The presence of proline at position 8 might be responsible for this different orientation of strand β 1, a conformation of this peptide segment similar to that in the icosahedral enzymes would require ϕ, ψ angles (-88° , -20°) unfavorable for proline.

The second short insertion is located between two residues, which form the short strand β 6b in SLS (Fig. 3). The two additional residues result in the transformation of the rigid β -hairpin region into a more flexible loop with no stabilizing main-chain hydrogen bonds.

The pentamer

The pentameric contacts in MLS are basically the same as in SLS except for the loss of the interaction between the N-terminal strand and the β -sheet of an adjacent subunit (Fig. 2B). In the $F_o - F_c$ electron density map, a strong spherical electron density was found between subunits at the pentamer interface. We interpret this difference density as a sulfate ion because MLS was crystallized in the presence of ammonium sulfate. The oxygen atoms of the sulfate interact with the main chain NH group and the OG1 atom of Ser55 from one subunit and the guanidinium group of Arg190 from the adjacent subunit of the same pentamer.

Inhibitor binding to MLS

The architecture of the pyrimidine binding site in MLS and the mode of inhibitor binding are rather similar to SLS (Figs. 8, 9). Two conserved polar residues, Asn26 and Glu61, interact with their side chains with atoms of the inhibitor. The side chain of Asn26 is within van der Waals distance to a keto oxygen of the pyrimidine ring, and the carboxylic oxygen atoms of Glu61 form hydrogen bonds to two ribityl hydroxyl groups. The invariant His127 (His88 in SLS and BLS) is located closer to the inhibitor than in SLS and forms a hydrogen bond with its nitroso oxygen atom. All other hydrogen bonds of the substrate analogue to protein atoms involve main-chain atoms. The second keto group of the pyrimidine ring interacts with the NH of Ser59. The other ribityl hydroxyl groups form two hydrogen bonds with main-chain atoms of Phe152 from strand β 5 of the neighboring subunit and with Trp60 (NH). The indole moiety of residue Trp25 stacks with the pyrimidine ring of the inhibitor in a similar way as the side chain of Phe23 in SLS and BLS. However, the indole moiety is sandwiched between the

inhibitor and residues Gly5–Pro6 from the adjacent subunit, whereas the benzene ring of Phe23 of SLS is facing the trimer pore and more accessible to the solvent.

The second substrate binding site

The location of the binding site for the second substrate, 3,4-dihydroxy-2-butanone-4-phosphate, is not exactly known since there is no structural information available for complexes between this substrate and lumazine synthase. In MLS, crystallized in the presence of ammonium sulfate, strong spherical electron density was observed in the vicinity of the first substrate (Fig. 8). This difference electron density was interpreted as a bound sulfate ion and might indicate the binding site for the phosphate group of the carbohydrate substrate, 3,4-dihydroxy-2-butanone-4-phosphate. This site coincides with the position of a bound phosphate ion in the

Fig. 9. The inhibitor binding pocket in lumazine synthase. Hydrogen bonds (<3.2 Å) and van der Waals contacts (<3.8 Å) are shown. Conserved residues involved in interactions with the inhibitor are shown in bold.

structure of BLS (Ritsert et al., 1995). The sulfate ion forms hydrogen bonds with the OG1 atom of the conserved residues Thr125 (Thr86 in SLS) and Arg166 (corresponding to Arg127 of SLS), as well as to the main-chain nitrogen of Glu124. The sulfate ion is close (~ 4 Å) to His127 (His88 in SLS) and to His176 (Lys135 in SLS). A model of the second substrate DHBP (Fig. 1) can be docked into the active site in such a way that both histidine side chains form hydrogen bonds with substrate oxygens. In this model, the side chain of His127 is located between the two substrates suggesting a central role of this residue in substrate binding and/or catalysis.

Discussion

The structural basis of assembly in lumazine synthases

The comparison of the three-dimensional structures of lumazine synthase from spinach, *B. subtilis*, and *M. grisea* shows that the overall subunit arrangement within the pentamers is rather similar despite poor conservation of the residues participating in the pattern of hydrogen bonding and salt bridges at this interface (Fig. 3). The only invariant hydrogen bond in the pentamer interface is found between two residues in the active site, His88 and Thr117 (SLS numbering). However, none of the substitutions results in a change of the main-chain conformation of residues at the interface. The similar packing of subunits into pentamers without conservation of the participating hydrogen bonds/salt bridges indicates that the complementarity of the interface surfaces plays a crucial role in the pentamer formation.

Figure 3 indicates that hydrogen bonds at the trimeric contact regions are also not conserved. However, it is not likely to be the reason for the inability of MLS to form an icosahedron, since residues participating in the trimeric contacts are not conserved even among capsid forming proteins. The crucial disturbance of this interface and thus the major cause for the failure to assemble into icosahedral capsids for the enzyme from *M. grisea* appears to be the two-residue insertion at the N-terminal part of the polypeptide chain. The insertion of Pro8–Gln9 results in a different orientation of the N-terminal strand $\beta 1$, causing sterical clashes with residues of neighboring subunits forming the trimer interface, in particular with the turn between helices $\alpha 4$ and $\alpha 5$ (Fig. 7), thus preventing proper assembly of trimers and consequently, preventing formation of icosahedral capsids.

Additional unfavorable interactions at this interface are introduced through the insertion of two residues, Ser173 and His174, in the β -hairpin between helices $\alpha 4$ and $\alpha 5$. In *M. grisea*, the loop in its present conformation would clash with a conserved asparagine residue in the substrate binding pocket of a neighbor related by the threefold axis. A similar four-residue insertion in this loop in the yeast enzymes has been suggested to interfere with the packing at this threefold axis, thus inhibiting the formation of icosahedral capsids (Mörtl et al., 1996). Superposition of the fungal enzyme on spinach lumazine synthase shows that already the insertion of only two residues in the hairpin between $\alpha 4$ and $\alpha 5$ indeed results in a disturbance of the contacts (Fig. 7).

The two-residue insertion at the N-terminus and the resulting conformational difference in the position of strand $\beta 1$ as the primary cause of the difference in assembly is supported by two other observations. First, the severe steric hindrance at the trimer interface also occurs in the absence of the two-residue insertion in the β -hairpin loop. Second, the available crystal structures of lumazine

synthase suggest that obstruction of the interface by residue insertion in the β -hairpin alone could be compensated by conformational changes of this loop. This notion is supported by findings with lumazine synthase from *Brucella abortus*. This enzyme lacks the insertion at the N-terminus, but has an insertion of three residues in the hairpin region at position 132–134. Nevertheless, in the crystal this enzyme seems to form icosahedral capsids, while it is a pentamer in solution (Goldbaum et al., 1998). Thus, while the three-residue insertion destabilizes the icosahedral packing, the ability to form the capsid is not completely lost.

Conserved amino acid residues in lumazine synthases

An alignment of the amino acid sequences of lumazine synthase from 20 different organisms is shown in Figure 3. The enzymes share 18–93% identity in pairwise comparisons. The most divergent lumazine synthases are found in the archaebacterium *Methanococcus jannaschii* and in *B. abortus*. Multiple alignment programs clustered the three plant enzymes with 70–75% of identity, whereas they exhibit 21–49% identity to the bacterial or fungal enzymes. Another cluster contains all bacterial lumazine synthases and a third cluster includes three lumazine synthases from yeast and fungi.

The alignment shows that there are only four invariant residues, Gly55, Gly84, Pro110, and Gly136. In addition, there are 15 highly conserved residues ($>90\%$) i.e., that differ in only one or two of the most distant sequences (Table 2). Lumazine synthase contains a surprisingly large proportion of conserved glycines and prolines. Out of 16–18 glycine residues, 10 are very conserved ($>85\%$ of the known sequences). All three prolines of bacterial LS (two in spinach) are conserved.

Inspection of the structure shows that the conserved glycine residues are required to maintain fold and active site architecture of the enzyme. Two conserved glycines, Gly55 and Gly79, are located in the C-termini of β -strands in the active site. Gly55

Table 2. Conserved residues in lumazine synthases^a

Residue	Variability	Proposed function
Asn24	His	Substrate-1 binding
Leu31	Ala, Met	Part of hydrophobic cluster
Gly34	Ser, Val	Packing of helices $\alpha 1$ and $\alpha 5$
Pro54	Leu	Active site loop
Gly55	—	Active site loop
Glu58	Asp	Substrate-1 binding
Gly79	Ala	Part of active site cleft
Ile82	Val	Part of active site cleft
Gly84	—	Trimer and pentamer contacts
Thr86	Ser, Ile	Substrate-2 binding
His88	Asp	Substrate binding
Pro110	—	Structural; part of tight turn
Gly114	Ser, Val	Packing of helix $\alpha 5$ with strand $\beta 5$
Thr117	Gly	H-bond with His88
Gln123	Ala, Ser	Pentamer contact
Ala124	Lys	Part of hydrophobic cluster
Arg127	His	Substrate-2 binding
Gly129	Phe	Intersubunit interaction at trimer interface
Gly136	—	Packing of helices $\alpha 1$ and $\alpha 5$

^aNumbering according to SLS.

(strand $\beta 3$) is located very close to the inhibitor, the distance between $C\alpha$ and O9 is only 3 Å. Absence of a side chain permits the hydrogen bonds between NH56 and O2, and NH57 and O9. Gly79 located on strand $\beta 4$ allows formation of the hydrogen bond between the carbonyl oxygen atom of residue 80 and the N3 atom of the inhibitor. Without these two glycines, the C-terminal ends of strands $\beta 3$ and $\beta 4$ could not come close enough to each other to form the main chain hydrogen bonds with the inhibitor.

The other conserved glycines are located further away from the active site. The helices $\alpha 1$ and $\alpha 5$ are almost perpendicular to each other and contacts between these two helices are made by the regions Gly34–Ala35 and Gly136–Ala137 with almost parallel planes of peptide bonds. The tight packing of strand $\beta 5$ on helix $\alpha 5$ is due to Gly114, which is very close to Ser139/Trp180 in SLS/MLS. Only one of the conserved glycines, Gly129, has ϕ, ψ angles in the region unfavorable for residues with side chains and the conformation of Gly129 is important for the intersubunit hydrogen bond at the trimer interface.

Inhibitor binding

Not all residues participating in binding of the inhibitor are invariant; however, the substitutions are mostly very conservative. The pyrimidine ring of the inhibitor is stacked with Phe23, which is substituted by Trp in yeast and fungi. Three residues, Asn24, and Glu58 form hydrogen bonds with the inhibitor by their side chains. Asp replaces Glu58 in *M. jannaschii*, and Asn24 is present in all LS sequences but two, *Mycobacterium tuberculosis* and *B. abortus* where it is substituted by a histidine. His88 is an invariant residue in all LS; it is aligned with Asp in *B. abortus*; however, the next residue is histidine. All residues that form the hydrophobic part of the inhibitor binding cleft are either conserved or substituted by other hydrophobic residues; Val82 (Ile in all other LS) and Val92 (Ile in yeast and fungi). The putative binding site for the second substrate also contains highly conserved residues: Thr86 (Ser in *Eremothecium*), Thr117 (Gly in *M. jannaschii*), and Lys135 (His in fungi and yeast).

A noteworthy feature of the protein-inhibitor interactions in LS is the relatively large number of hydrogen bonds formed by main-chain atoms compared to interactions involving protein side chains. For instance, of the eight hydrogen bonds between protein and inhibitor in *M. grisea*, five involve main-chain atoms of the enzyme (Fig. 9).

Materials and methods

Purification of spinach LS

The gene coding for the mature spinach LS was cloned and expressed in *E. coli* BL21(DE3). The cloning of the spinach LS gene by functional complementation of an *E. coli* auxotroph, protein expression, and characterization of SLS will be described in details elsewhere (D. Jordan & P. Viitanen, unpubl. obs.). The enzyme used in the crystallographic studies was purified to homogeneity in two steps. First, the cell-free extract was applied to a Mono-Q HR 10/10 column (Pharmacia Biotech Inc., Piscataway, New Jersey), pre-equilibrated with 50 mM Tris-HCl, pH 7.6, 10 mM sodium sulfite, and 1 mM EDTA. The protein was eluted with a NaCl gradient (0–0.5 M). The enzyme was concentrated to 22 mg/mL and further purified on a TSK G3000SW gel filtration column in

50 mM Tris-HCl, pH 7.7, and 0.3 M NaCl. Pure spinach LS eluted as a single peak. The protein was concentrated to a final concentration of ~20 mg/mL in 30 mM Tris pH 7.5, containing 10 mM of the inhibitor, 5-nitroso-6-ribitylamino-2,4-pyrimidinedione.

Cloning, expression, and purification of *M. grisea* LS

A detailed description of the cloning, expression, and purification of *M. grisea* LS will appear elsewhere (D. Jordan, P. Viitanen, unpubl. obs.). Briefly, a cDNA insert encoding the full-length protein was identified through functional complementation of an *E. coli* LS knockout mutant essentially as for spinach LS (D. Jordan & P. Viitanen, unpubl. obs.). The cDNA expression library was prepared from isolated mRNA using conventional methodologies and ligated into the EcoRI and XhoI sites of pBluescript II SK (+) (Stratagene, La Jolla, California). For expression in *E. coli*, the ORF of *M. grisea* LS was amplified by polymerase chain reaction (PCR) using Primers 1 and 2 to modify its 5' and 3' ends for insertion into the high-level *E. coli* expression vector pET-24a (+); this manipulation had no effect on the primary amino acid sequence. Primer 1 (CTACTCATTTCatagCACACCAAAGGCCCGA) introduces an NdeI site at the initiator Met residue, while Primer 2 (CATCTTACTGGATCC ATCACTCG GTCTTCCCGGC) provides a unique BamHI site just past the stop codon. The resulting PCR fragment was cut with NdeI and BamHI and ligated into similarly digested pET-24a (+) (Novagen, Madison, Wisconsin) to yield the plasmid pET24MGLSB1; the latter was sequenced completely to confirm the absence of PCR errors.

E. coli BL21(DE3), transformed with pET24MGLSB1, was grown in LB medium that contained 50 µg/mL kanamycin (37 °C). Induction was for 3 h with isopropyl-1-thio- β -D-galactopyranoside, added at an A_{600} nm of ~0.9. Subsequent steps were at 0–4 °C. Cells were disrupted in three volumes of 50 mM Tris-HCl, pH 7.5, by using two passes through a microfluidizer (model 110Y, Microfluidics Corp., Newton, Massachusetts). Following centrifugation (40,000 g, 20 min), the supernatant was adjusted to 25% saturation with ammonium sulfate and centrifuged as above. The supernatant was applied to a Phenyl-Sepharose (Pharmacia, Uppsala, Sweden) column equilibrated in 50 mM Tris-HCl, pH 7.5, and 25% saturated ammonium sulfate. The column was developed in the same buffer with a linear gradient (25% saturated ammonium sulfate to 0%). Active fractions were desalted and applied to a Q-Sepharose Fast Flow (Pharmacia) column equilibrated with 50 mM Tris-HCl, pH 7.5. The column was developed with a linear gradient (0–0.5 M NaCl) in the same buffer. Active fractions were concentrated and diafiltered to yield a final concentration of 25 mg/mL in 50 mM Tris-HCl, pH 7.5, containing 35 mM 5-nitroso-6-ribitylamino-2,4-pyrimidinedione. The protein protomer was characterized by electrospray ionization mass spectrometry to be of the expected mass. Sedimentation equilibrium studies indicated that the protein exists as a pentamer. The $V_{\max}$ of MLS was ~3 min⁻¹ at 25 °C or about three times faster than other LS enzymes that have been characterized.

Crystallization

Crystallization experiments were carried out at room temperature by hanging drop vapor diffusion. SLS was crystallized in 0.1 M Tris-HCl pH 7.6, 17% PEG 1500, and 5% PEG 400. MLS was crystallized in 100 mM MES pH 6.5, 27% PEG monomethyl ether 5000, and 200 mM ammonium sulfate. The protein concentrations

were 22 and 6 mg/mL, respectively, containing 10 mM of the inhibitor, 5-nitroso-6-ribitylamino-2,4-pyrimidinedione.

Data collection

Data were collected at 100 K using a MAR image plate, X-ray Research, Hamburg at beam line BL711, MAX Laboratory (Lund, Sweden). The crystals were quickly soaked in crystallization buffer supplemented with 20% PEG 400 (SLS) and 20% ethylene glycol (MLS), respectively, before flash freezing in liquid nitrogen. One crystal was used for the collection of each data set.

Determination of space group and cell dimensions was achieved using the auto-indexing option in DENZO and by examination of pseudo-precession images obtained using the program PATTERN (Lu, 1996). The crystals of the spinach enzyme belong to the cubic space group $P2_13$ with cell dimensions $a = b = c = 218.3$ Å, and the MLS crystals belong to space group $P2_12_12_1$ with cell dimensions $a = 98.8$ Å, $b = 124.7$ Å, and $c = 141.4$ Å. The data were integrated and scaled using DENZO and SCALEPACK (Otwinowski, 1993). Data collection statistics are shown in Table 1.

Structure solution and refinement

The SLS structure was solved by molecular replacement using the program AMoRe (Navaza, 1994). A dimer of pentamers, constructed from the coordinates of lumazine synthase from *B. subtilis* (Protein Data Bank code 1rvv), was used as the starting model. The inhibitor was not included in the search model. The rotation function calculation was performed between 20 and 4 Å resolution and resulted in long list of peaks due to the many possible ways of arranging the pentamers. The 40 highest peaks were used for a translation search giving correlation coefficients ranging from 29.2 to 22.6. After the fit function, the highest peaks had correlation coefficients between 33.7 and 28.6. The different solutions were examined using the program O (Jones & Kjeldgaard, 1997) to distinguish between pentamers that were related by crystallographic and noncrystallographic symmetry. The asymmetric unit was found to contain four pentamers, which corresponds to a V_m value equal to 2.6 Å³/Da (Matthews, 1968). Before any refinement was initiated, all residues that differed from the starting model were exchanged for alanines.

In the first steps of the rigid body refinement, the 20 subunits were treated as separate groups. Strict icosahedral symmetry (five-fold, threefold, and twofold rotations) was applied, and the whole particle was then refined by further rigid body minimization. Electron density maps were improved by 20-fold noncrystallographic averaging, solvent flattening, and histogram matching with the program DM (Cowtan, 1994). The averaged map was easily interpretable; the sequence could be fitted to the electron density and the model rebuilt where necessary using the graphics program O (Jones & Kjeldgaard, 1997). Already at that stage, electron density for the inhibitor was clearly visible in the averaged map, but the inhibitor was not added until a round of simulated annealing was performed. The model was further refined by several cycles of rebuilding and positional refinement including overall B -factor refinement and a bulk solvent correction. All refinement was carried out in X-PLOR (Brünger et al., 1990) applying strict noncrystallographic symmetry. To monitor the progress of refinement, 4% of the data was set aside for the calculation of R_{free} (Brünger, 1992). The parameters described by Engh and Huber were used (Engh & Huber, 1991), and the dictionary for the inhibitor was generated

using XPLO2D (Kleywegt & Jones, 1997). Due to the low resolution of the electron density map, no solvent molecules were added to the model. The final R_{free} and R_{cryst} for the model refined with strict icosahedral symmetry is 33.0 and 32.0%, respectively. The quality of the model was analyzed with PROCHECK (Laskowski et al., 1993). The RMSDs for bonds and angles are 0.014 and 1.713 Å. In the last rounds of positional refinement, tightly restrained NCS was applied. This yielded a R_{free} and R_{cryst} of 32.6 and 31.0%, which reflects the poor observation-to-parameter ratio at this moderate resolution.

The structure of MLS was solved by molecular replacement but with only one BLS pentamer as search model. The asymmetric units contain two pentamers corresponding to a V_m value of 2.0 Å³/Da. The first 20 solutions from the rotation function were used in the translation function giving correlation coefficients ranging from 22.6 to 20.2. Next, the first peak was fixed and the translation function was rerun after which a peak, slightly higher than the other peaks, was found (correlation coefficients 28.4). After the fit function, the correlation coefficients for the two solutions increased to 37.9. Refinement of the MLS complex was carried out using a similar protocol as for the SLS structure. Five percent of the data was used for monitoring R_{free} and tightly restrained NCS was used throughout the refinement. The residues differing between the search model and the MLS enzyme were exchanged for alanines after which a 10-fold averaged map was calculated. The averaged map was of good quality, and the model could be rebuilt with the correct sequence. After simulated annealing, the inhibitor could be unambiguously modeled into the positive difference electron density at the binding site, and a sulfate ion bound to the potential second substrate binding site was included in the model (Fig. 8). In a last cycle, the model was further refined with CNS (Brünger et al., 1998). The final model consists of 10 subunits, each comprising 165 residues, 10 inhibitor molecules, 19 sulfate ions, and 10 water molecules. The values of the final R_{cryst} and R_{free} are 24.8 and 27.1%, respectively. Details of the refinement statistics are given in Table 1.

The nucleotide sequence of lumazine synthase from *M. grisea* has been deposited to GENBank, accession number AF148449. The atomic coordinates for spinach and *M. grisea* lumazine synthase have been deposited with the Protein Data Bank, accession numbers 1c2y and 1c41, respectively.

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