High Order Quaternary Arrangement Confers Increased Structural Stability to Brucella sp. Lumazine Synthase*

Received for publication, November 3, 2003, and in revised form, November 26, 2003
Published, JBC Papers in Press, December 1, 2003, DOI 10.1074/jbc.M312035200

Vanessa Zylberman‡‡, Patricio O. Craig‡, Sebastián Klinko‡‡, Bradford C. Braden†, Ana Cauerhff‡ and Fernando A. Goldbaum‡‡‡

From the Instituto Leloir, Consejo Nacional de Investigaciones Científicas y Técnicas and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Patricia Argentinas 435 (C1405BWE), Buenos Aires, Argentina and the Department of Natural Sciences, Bowie State University, Bowie, Maryland 20715

The penultimate step in the pathway of riboflavin biosynthesis is catalyzed by the enzyme lumazine synthase (LS). One of the most distinctive characteristics of this enzyme is the structural quaternary divergence found in different species. The protein exists as pentameric and icosaedral forms, built from practically the same structural monomeric unit. The pentameric structure is formed by five 18-kDa monomers, each extensively contacting neighboring monomers. The icosaedralic structure consists of 60 LS monomers arranged as 12 pentamers giving rise to a capsid exhibiting icosaedral 532 symmetry. In all lumazine synthases studied, the topologically equivalent active sites are located at the interfaces between adjacent subunits in the pentameric modules. The Brucella sp. lumazine synthase (BLS) sequence clearly diverges from pentameric and icosaedral enzymes. This unusual divergence prompted us to further investigate its quaternary arrangement. In the present work, we demonstrate by means of solution light scattering and x-ray structural analyses that BLS assembles as a very stable dimer of pentamers, representing a third category of quaternary assembly for lumazine synthases. We also describe by spectroscopic studies the thermodynamic stability of this oligomeric protein and postulate a mechanism for dissociation/unfolding of this macromolecular assembly. The higher molecular order of BLS increases its stability 20 °C compared with pentameric lumazine synthase. The decameric arrangement described in this work highlights the importance of quaternary interactions in the stabilization of proteins.

Ribbonavin, an essential cofactor for all organisms, is biosynthesized in plants, fungi, and microorganisms. The penultimate step in the pathway is catalyzed by the enzyme lumazine synthase (LS).1 One of the most distinctive characteristics of this enzyme is the structural quaternary divergence found in different species. The protein exists as pentameric and icosaedral forms, built from practically the same structural monomeric unit. The structure of the monomer consists of four repeated β-strand/α-helix motifs producing a sandwich of four parallel β-strands surrounded by four α-helices, two on each face of the β sheet (1). In all LS studied, the topologically equivalent active sites are located at the interfaces between adjacent subunits in the pentameric modules (2).

Bacillaceae express a bifunctional enzyme complex with lumazine synthase and riboflavin synthase activity. Three α-subunits (riboflavin synthase) enclosed by 60 β-subunits (lumazine synthase) form a protein particle of ~1 MDa (1, 3). The three-dimensional structure of the lumazine synthase/riboflavin synthase from Bacillus subtilis complexed with a substrate analogue has been determined (4). This structure consists of 60 β-subunits (lumazine synthase monomers) arranged as 12 pentamers giving rise to a capsid exhibiting icosaedral 532 symmetry. Two other icosaedral LS have been described by x-ray crystallography. Spinach and thermophilic bacterial Aquifex aelicus LS also exhibit icosaedral 532 symmetry. The three proteins form 1-MDa spherical capsids of 60 LS subunits with icosaedral symmetry. The icosaedral LS structures superimpose very well, highlighting the conservation of the overall folding and quaternary arrangement despite the low sequence homology between them (5).

Four structurally characterized LS as pentamers in their native form and do not further associate to form an icosaedral capsid. These include fungal Magnaporthe grisea, yeast Saccharomyces cerevisiae, and Schizosaccharomyces pombe and bacterial Brucella abortus LS (5). Although the four pentameric enzymes fold in a similar arrangement, the postulated reasons for the lack of icosaedral order differ. Superposition of the pentameric LS shows that the loop connecting the helices α4 and α5 is critical for preventing the formation of capsids. In all icosaedral LS a pentapeptide kink located in this loop is essential for pentamer-pentamer contacts (5, 6). The yeast and fungal pentameric enzymes have insertions of different length in this loop that change its overall orientation and disrupt potential contacts to neighboring subunits (7). We have previously analyzed the divergence in macromolecular assembly between pentameric and icosaedral LS enzymes. In this regard, a high degree of divergence was observed between the sequence from B. abortus LS and the other pentameric structurally characterized members of the family (8). BLS has a 3-residue insertion between helices α4 and α5 that contribute to form a continuous undistorted helix, unable to form the extrapolation method; GdnHCl, guanidine hydrochloride; Mw, weight average molecular weight; DTT, dithiothreitol; ASA, accessible surface area.
capsid-stabilizing kink (6). Thus, the BLS structure clearly diverges from pentameric and icosahedral enzymes because of the lack of this critical loop. The different orientation of this straight helix is compensated by a longer loop bridging the helix structure with the contiguous sheet. This unusual divergence prompted us to further investigate the quaternary arrangement of BLS. In the present work, we demonstrate by means of solution light scattering and x-ray structural analysis that BLS assembles as a very stable dimer of pentamers, representing a third category of quaternary assembly for LS. We also describe, by spectroscopic studies, the thermodynamic stability of this oligomeric protein and postulate a mechanism for dissociation/unfolding of its macromolecular assembly.

EXPERIMENTAL PROCEDURES
Expression of the LS Protein

The Brucella sp. LS gene was cloned in pET11a vector (Novagen) as reported previously (9). The plasmid was used to transform BL21(DE3)<sup>®</sup> strain Escherichia coli-competent cells (Stratagene, La Jolla, CA). Ampicillin-resistant colonies were grown until A<sub>600</sub> = 1.0 in LB medium containing 100 μg/ml ampicillin, at 37 °C with agitation (300 rpm). Five milliliters of this culture was diluted to 500 ml and grown to reach an A<sub>600</sub> of 1.0. At this point the culture was induced adding 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubated for 4 h at 37 °C with agitation (300 rpm). The bacteria were centrifuged at 15,000 × g during 20 min at 4 °C.

Protein Purification and Refolding

BLS protein was successfully expressed as inclusion bodies by transformation of strain BL21(DE3)<sup>®</sup> E. coli-competent cells (9). The inclusion bodies were solubilized in 50 mM Tris, 5 mM EDTA, 8 mM urea pH 8.0 at room temperature overnight with agitation. The solubilized material was refolded by dialysis during 72 h against phosphate-buffered saline containing 1 mM dithiothreitol. This preparation was purified in a Mono-Q column in a fast-protein liquid chromatography apparatus (Amersham Biosciences, Uppsala, Sweden) using a linear gradient of buffer B (50 mM Tris, 1 mM NaCl, pH 8.5). The peak enriched with BLS was further purified on a Superdex-200 column with phosphate-buffered saline buffer, 1 mM DTT. The purity of the BLS preparation was determined on SDS-15% (w/v) polyacrylamide gels. Purified BLS was concentrated (10 mg/ml), frozen in liquid N<sub>2</sub>, and stored at −20 °C.

Circular Dichroism

BLS samples were diluted in 50 mM sodium phosphate, pH 7.0, 1 mM DTT with a concentration of denaturants (urea or GdnHCl). All experiments were performed at 25 °C, and samples were incubated at least 2 h before taking CD measurements. Spectra were measured on a spectropolarimeter (JASCO J-810) using either 0.1- or 0.5-cm path length quartz cells. Unfolding was monitored by far-UV CD (260

Thermal Denaturation Monitored by CD

BLS samples were incubated in 50 mM sodium phosphate, 1 mM DTT, pH 7.0 (in the presence or absence of 2 M GdnHCl) or in 50 mM buffer citrate, 1 mM DTT, pH 4.5. Thermal denaturation was conducted by slowly increasing the temperature with a Peltier system (Jasco). The range of temperature scanning was 25–95 °C at a speed of 4 °C/min. Molar ellipticity at 222 nm was measured every 0.5 °C. Fast or slow cooling back to 25 °C (from 95 to 25 °C at a speed of 1 °C/min) did not show a recovery of ellipticity demonstrating the irreversibility of the thermal unfolding. Thus the temperature midpoint of the thermal transition was considered as an apparent T<sub>μ</sub>.

pH Dependence of LS Unfolding

pH-induced BLS dissociation and unfolding was evaluated by diluting protein samples (5 μM monomers) in 50 mM citrate, 1 mM DTT, pH 6.0–2.5 or in 50 mM sodium phosphate, 1 mM DTT, pH 7.5–7.0. After 1–2 h of incubation at room temperature, CD and SLS signals were determined. ANS fluorescence emission was measured in the same buffer conditions as CD and SLS experiments, aggregating to the samples 50 μM ANS. Values were normalized to ANS fluorescence emission in the same buffer. Excitation and emission were set at 365 and 470 nm, respectively.

Determination of Molecular Weight of BLS by Static Light Scattering

The weight average molecular weight (M<sub>ω</sub>) of BLS under different conditions was determined on a Precision Detectors PD2010 light-scattering instrument tandemly connected to an high-performance liquid chromatography system and an LKB 2142 differential refractometer. In general, 20–100 μL of LS (0.3–1 mg/ml) was loaded on a Superdex 200 HR-10/30 (24 ml) or a Sephadex G-25 (1 ml) column and eluted with 50 mM phosphate buffer phosphate, 1 mM DTT under different pH, urea, GdnHCl, and NaCl conditions. The 90° light scattering and refractive index signals of the eluting material were recorded on a PC computer and analyzed with the Discovery32 software supplied by Precision Detectors. The 90° light scattering detector was calibrated using bovine serum albumin (M<sub>ω</sub>: 66.5 kDa) as a standard. Prior to the injection in a size exclusion chromatography column (SEC), each sample was preincubated for 1–2 h at room temperature in the elution buffer.

Thermodynamic Parameters

Thermodynamics evaluation of GdnHCl-induced unfolding of BLS was fitted to a two-step model: N<sub>10</sub> ↔ U(1) ↔ U(2) ↔ 5U. The first step (N<sub>10</sub> ↔ 2N<sub>5</sub>) represents the dissociation of decameric BLS (N<sub>10</sub>) in two folded pentamers (N<sub>5</sub>), whereas the second step (N<sub>5</sub> ↔ 5U) represents the concomitant dissociation and unfolding of the pentameric structure (N<sub>5</sub>) in monomeric subunits (U). Because both steps are well resolved from each other, their thermodynamic parameters were independently analyzed using the linear extrapolation method (Equation 1) assuming a two-state transition model for each step (10),

\[ \Delta G_U = \Delta G_{10} - m[D] \]  
where \( \Delta G_U \) is the free energy of unfolding of a protein at a given denaturant concentration, \( \Delta G_{10} \) is the free energy of unfolding in the absence of denaturant, and \( m \) is the dependence of the free energy on denaturant concentration (\( [D] \)).

Step 1—The first step was monitored by SLS in the range of 1.5–2.2 M GdnHCl. The concentration at equilibrium of the N<sub>10</sub> and N<sub>5</sub> species of BLS were calculated using Equations 4 and 5 derived from rearrangement of Equations 2 and 3,

\[ M_{\omega} = M_{\omega}(ccN_{10} + M_{\omega}(ccN_{5}))/ccT \]  
(2)

\[ ccT = ccN_{10} + ccN_{5} \]  
(3)

\[ N_{10} = ccT - ccN_{5}M_{\omega} \]  
(4)

\[ N_{5} = (ccT - ccN_{10}M_{\omega})/(ccN_{5} + ccT) \]  
(5)

where \( M_{\omega} \) represents the weight average molecular weight of BLS, ccN<sub>10</sub>, ccN<sub>5</sub>, and ccT represent the decamer, pentamer, and total protein concentration in milligrams/ml, respectively, and \( N_{10} \) and \( N_{5} \) represent the molar concentration and molar weight of the decameric (174.4 kDa) and pentameric (87.2 kDa) species of BLS, respectively.

The equilibrium constant K<sub>G10</sub> and the free energy change \( \Delta G_{G10} \) for Step 1 are defined in Equations 6 and 7.

\[ K_{G10} = [N_{10}]^{2}/[N_{5}] \]  
(6)

\[ \Delta G_{G10} = -RT \ln K_{G10} \]  
(7)

The \( \Delta G_{G10}, \) and \( m \), values (Equation 1) for this transition were obtained from the extrapolation to zero denaturant concentration and from the slope of the linear regression fit of the \( \Delta G_{G10}, \) values calculated.
as a function of GdnHCl concentration in the range of 1.5 to 2.2 M. The dissociation constant of the decameric arrangement \( K_1 \) was estimated from the \( \Delta G_{U2}^{\text{eo}} \) value of this transition using the equation, 
\[
K_1 = e^{-\frac{\Delta G_{U2}^{\text{eo}}}{RT}}
\]

Step 2—The second step was monitored by CD and FT in the range of 2.4 and 3.5 M of GdnHCl. The equilibrium constant \( K_{U2} \) and the free energy change \( \Delta G_{U2} \) for this transition are defined in Equations 8 and 9.

\[
K_{U2} = \frac{[U]^5}{[N_5]^5} \quad \text{(Eq. 8)}
\]

\[
\Delta G_{U2} = -RT \ln K_{U2} \quad \text{(Eq. 9)}
\]

The total protein concentration in monomer units \( P_2 \) and the fractional population in the native \( (F_N) \) and unfolded \( (F_U) \) states were calculated using Equations 10–12,

\[
P_2 = 5[N_5] + [U] \quad \text{(Eq. 10)}
\]

\[
F_N = 5[N_5]/P_2 \quad \text{(Eq. 11)}
\]

\[
F_U = 1 - F_N = [U]/P_2 = (m_1[D] + F) - Y[(m_1[D] + F) - (m_2[D] - U)] \quad \text{(Eq. 12)}
\]

where \( Y \) is the experimental spectroscopic value, \( [D] \) is the GdnHCl concentration, \( F \) and \( U \) are the intercepts, and \( m_1 \) and \( m_2 \) are the slopes of the pre- and post-unfolding baselines, respectively. Combining Equations 1 and 8–12, we obtain the general equation (Equation 13) as follows.

\[
\frac{m_1[D] + F - Y}{(m_1[D] + F) - (m_2[D] + U)} P_2^5 + \left[ \frac{(m_1[D] + F - Y)}{(m_1[D] + F) - (m_2[D] + U)} - 1 \right] e^{\frac{\Delta G_{U2}^{\text{eo}}}{RT}} = 0 \quad \text{(Eq. 13)}
\]

\( \Delta G_{U2}^{\text{eo}} \) values of Step 2 were obtained fitting the experimental data measured by CD and FT to Equation 13 by a non-linear square fit method. The midpoint of this transition \( [D]_{50\%} \) was calculated as in Equation 14.

\[
[D]_{50\%} = \left[ RT \ln(5/16 P_2^5) + \Delta G_{U2}^{\text{eo}}/m \right] \quad \text{(Eq. 14)}
\]

**Crystallization, Data Collection, and Structure Determination**

In addition to the previously obtained BLS crystals, which led to the resolution of its three-dimensional structure (6), two further crystal forms of BLS were obtained in this work by means of the hanging drop, vapor diffusion method. The first form was diamond-like crystals obtained using 30% (w/v) polyethylene glycol 400, 0.1 M sodium acetate in 0.1 M MES buffer, pH 6.5, which diffracted to 2.9 Å and belongs to the trigonal space group \( P_3_2 1 \). Furthermore, we obtained plate-like crystals using 12% (w/v) polyethylene glycol 4000, 0.1 M sodium acetate in 0.1 M HEPES buffer, pH 7.5, which diffracted to 3.0 Å and belongs to the monoclinic space group \( P_2_1 \). X-ray diffraction data were collected both at our in-house x-ray source, a Bruker M18XH6 MAC Science rotating anode interfaced to a Siemens X-1000 multiframe area detector and at the D03B protein crystallography beamline at the Laboratorio Nacional de Luz Sincrotrón, Campinas, Brazil (11). Data reduction and processing were carried out with the programs MOSFLM, Scala, and Truncate from the CCP4 suite (12). Crystal packings were determined using the molecular replacement procedure as implemented in the AMoRe package (12), with the previously solved BLS structure as a search model. Crystallographic symmetry construction was carried out using the program O (13).

**Structural Analysis**

Total accessible surface area and buried surface areas of interaction (ASA) between monomers and pentamers in pentamer and decamer structures, respectively, were calculated with Surface Racer 1.2 program (14) using an implementation of the Lee and Richards (15) algorithm and a probe radius of 1.7 Å. Intermolecular polar and non-polar interactions were calculated with the Molmold2k2 (16) and Contacts of Structural Units (17) programs. Any pair of atoms is considered to be in contact if the distance between them is less than 4 Å.

**RESULTS**

**BLS Is a Stable Dimer of Pentamers in Solution**—Previous studies (6, 9) have shown that BLS does not assemble as a 1-MDa capsid oligomer, having a retention time in SEC compatible with an apparent molecular mass of 90 kDa. This behavior suggested that the oligomeric structure of this protein in solution was a pentamer. However, the estimation of the molecular weight of a protein by its retention time on a SEC column is a method prone to artifacts. Additionally, the sequence divergence of BLS, compared with other pentameric LS, prompted us to re-analyze the quaternary structure of the protein by spectroscopic techniques such as static light scattering (SLS).

SLS experiments show that the protein has a molecular mass...
of 180 kDa in solution, corresponding to an assembly of two pentamers (decameric arrangement of the 18-kDa polypeptide chain). To characterize this new quaternary arrangement, we studied the thermodynamic stability of BLS, evaluating the unfolding of the protein induced by the common chemical denaturants (urea and GdnHCl) and pH.

Preincubation of BLS with increasing concentrations of urea shows that the enzyme remains as a stable dimer of pentamers, with no detectable change in its quaternary structure (180 kDa, determined by SLS, data not shown) as well as in its tertiary and secondary structures as followed by tryptophan fluorescence and CD (Fig. 1). The absence of structural changes, even in 8 M urea, indicates that the quaternary arrangement of BLS is very stable.

Conversely, GdnHCl produces a cooperative and reversible change in the tertiary structure reflected by a decrease in tryptophan fluorescence emission (Fig. 1A). This measure senses the environment of Trp-22, the unique tryptophan in BLS monomers that is located on the active site at the monomer-monomer interface (6). In addition, GdnHCl incubation (6 M) produces a complete loss of secondary structure of BLS as monitored by CD spectra (Fig. 1B). The differential effect of GdnHCl and urea cannot be explained by their differences in ionic strength. The behavior of BLS in 8.0 M urea and in the presence of 1 M NaCl is superimposable with its described stability in absence of salt (data not shown), implying that unfolding with GdnHCl seems to be due to more specific interactions of the guanidinium cation with the protein (18, 19). Thus, we used GdnHCl-induced denaturation to study the mechanism of BLS unfolding.

Dissociation of BLS Dimer of Pentamers Is Tight and Precedes the Unfolding of the Pentamer—SLS analysis of the GdnHCl-induced unfolding of BLS shows a biphasic behavior.
mediates BLS pentamer (concentration of GdnHCl required to achieve a 50% decrease in molar ellipticity). \([D]_{520}^\theta\) values were calculated using Equation 13.

\[ \Delta G = \Delta G^\circ + RT \ln \frac{[D]_{520}^\theta \text{at pH } 7.0}{[D]_{520}^\theta \text{at pH } 4.0} \]

Table 1

| Protein | \([D]_{520}^\theta\) | \[\Delta G\] |
|---------|-----------------|-------------|
| 0.25    | 2.50            | 322 ± 30    |
| 0.50    | 2.70            | 344 ± 27    |
| 2.50    | 2.70            | 330 ± 16    |
| 5.0     | 2.76            | 347 ± 18    |
| 25.0    | 2.88            | 317 ± 30    |

\(\Delta G^\circ\) is the free energy of unfolding of the intermediate BLS pentamer in the absence of denaturant. Values were derived from the average of three independent GdnHCl denaturation experiments monitored by CD spectroscopy. Free energies were determined by non-linear regression analysis (see “Experimental Procedures”).

(Fig. 2A). In the first step, observed between 1.5 and 2.2 M GdnHCl, the SLS signal intensity of the protein is reduced to half the value measured in the absence of denaturant. However, no changes in the far-UV CD spectra and tryptophan fluorescence of the protein are observed in this range (Figs. 1A and 2A). Thus, intrinsic tryptophan fluorescence is completely insensitive to the change in the quaternary structure of the protein, indicating that the dissociation does not modify significantly the environment of Trp-22. These results point to the existence of a dissociation phenomenon of the decameric structure of BLS into two pentameric subunits, with no further changes in tertiary and secondary structure. On the other hand, the second step observed between 2.4 and 3.5 M GdnHCl, shows a 5-fold decrease in the SLS signal of BLS concomitantly to the disappearance of its far-UV CD signal at 222 nm, and a significant decrease in the fluorescence intensity of its single tryptophan residue (Figs. 1A and 2A). These results are interpreted as an unfolding and loss of the tertiary and secondary structures of the protein coupled to the dissociation of its pentameric arrangement in five monomeric subunits (18 kDa). The overlapping of the changes observed in the second step by SLS, CD (Fig. 2A) and fluorescence (Fig. 1A) clearly supports our model. These results were further verified by SLS coupled to gel filtration chromatography (Fig. 2B), where it was possible to isolate a 90-kDa intermediate at 2 M GdnHCl. The fact that an intermediate of 90 kDa is stable enough to be detected by this methodology suggests that the decameric assembly is composed of two previously associated pentamers.

The dissociation of the decameric to folded pentamers is also observed when BLS is incubated at acidic pH (Fig. 3). This phenomenon is evidenced by a 2-fold decrease in the SLS signal intensity in the range of pH 4.0–5.0 as compared with the value measured at pH 7.0. ANS binds to exposed hydrophobic surfaces in partially folded intermediates with higher affinity than to native or completely unfolded proteins (20–22). This binding result in a marked increase in fluorescence emission compared with the free ANS. We found that the dissociation of BLS at pH in the range 6.0–4.0 is a reversible process that occurs without significant exposure of hydrophobic patches and changes in secondary structure as monitored by ANS fluorescence and CD spectra (Fig. 3).

At pHs below 4.0 the protein shows an irreversible unfolding, evidenced by a loss of ellipticity at 222 nm and a dramatic increase in binding to ANS. Clearly, both changes are coupled, implying that at acidic pHs the protein exposes hydrophobic surfaces when secondary structure is lost. The light scattering response cannot be accurately monitored below pH 4.0, presumably because of aggregation.

Thermodynamic Stability of BLS Measured by Chemical Denaturation.—To describe the thermodynamic stability of BLS, we studied the dissociation and unfolding steps taking advantage of the differential effect of GdnHCl at distinct concentration ranges. Both transitions (Steps 1 and 2) were shown to be highly cooperative and reversible. The dissociation step was characterized by SLS using GdnHCl up to 2.2 M, in a condition that does not disturb the tertiary and secondary structures of the pentamer. This assumption is supported by the facts that the circular dichroism spectrum is not modified (see Fig. 2A) and the intrinsic tryptophan fluorescence does not vary upon addition of GdnHCl (see Fig. 1A). The SLS determination of the equilibrium between the dimer of pentamers and the pentamer gives a \(\Delta G\) of 90 ± 20 kJ/mol decamer as estimated by the LEM method assuming a two-state transition (see “Experimental Procedures”). This value indicates that the protein remains as a decamer under physiological conditions (estimated \(K_d\) = 2.48 × 10^{-10} M).

On the other hand, the transition from folded pentamer to unfolded monomers is a highly cooperative process that can be measured by GdnHCl denaturation at higher concentrations followed by tryptophan fluorescence (Fig. 1A), SLS, and circular dichroism (Fig. 2A). These signals show a sharp and overlapping change around 2.4–3.5 M GdnHCl. Thus, all three spectroscopic analyses rule out the existence of a populated intermediate during this transition. The dependence of \([D]_{520}^\theta\) with protein concentration (Table I) clearly supports the model of a two-state transition from a folded pentamer to unfolded monomers. Thermodynamic analysis of this equilibrium shows that the \(\Delta G\) is 330 ± 30 kJ/mol pentamer (Table I; see “Experimental Procedures” for details). In agreement with this analysis, GdnHCl denaturation of BLS previously incubated at pH 5.0 (as a dissociated pentamer, see Fig. 3), gives a \(\Delta G\) of 300 ± 30 kJ/mol pentamer, clearly indicating the accuracy of the determined thermodynamic parameter for the stability of the pentamer.

Thermal Denaturation of BLS.—The stability of BLS to thermal denaturation was followed by measuring the molar ellipticity at 222 nm as a function of increasing temperature, as shown in Fig. 4. The enzyme shows a sharp decrease of ellipticity between 85 and 95 °C with an apparent \(T_m\) of 88 ± 2 °C. The loss of secondary structure is not recovered after slow cooling of the samples, indicating that an irreversible unfolding.
phenomenon of BLS takes place under these conditions. Thermal denaturation of the protein, previously incubated in two different conditions that produce the dissociation of the decamer into pentamers (2.0 M GdnHCl and pH 4.5), produces a very similar decrease of about 20°C in the thermal stability of BLS (see Fig. 4), further demonstrating that dissociation and denaturation can be uncoupled under these conditions. Thus, this shift of 20°C can be attributed to the contribution of the pentamer-pentamer interface to the overall stability of BLS.

**Quaternary Arrangement of BLS Is Confirmed by X-ray Crystallography**—X-ray structure analysis confirmed the quaternary arrangement of BLS. *Brucella* sp. LS has been crystallized in three different forms (Refs. 6 and 23 and this work). One of these forms (space group P3121) has a high content of water (around 70%) and diffracts x-rays to medium resolution (2.9 Å) with a single pentamer in the asymmetric unit. Modifying the reagent conditions we obtained a second crystalline form (space group P21) that has two pentamers in the asymmetric unit. The previously obtained crystal form (space group R32) is more densely packed and diffracted to 2.7-Å resolution with a single pentamer in the asymmetric unit, allowing for the resolution of the three-dimensional structure of the enzyme (RCSB Protein Data Bank code 1DIO). Despite the different types of crystal arrangement (Fig. 5A), all crystal forms show the same quaternary arrangement. The previously described pentamer forms a tightly packed dimer of pentamers (Fig. 5B), with the N terminus of the straight α helix (composed of helices α4 and α5, as named in other LS) and the loop connecting the contiguous sheet making a protuberant surface that tightly fits in the neighboring pentamer. This region of the polypeptide chain has a very high content of histidines and phenylalanines (7 His and 4 Phe on a stretch of 18 residues) (Fig. 5C).

Tables II and III show the structural analysis about the nature of the monomer-monomer and pentamer-pentamer interfaces. Each of the monomers that gives rise to the decameric structure buries 45% of its ASA, established mainly by hydrophobic contacts with two neighboring monomers (pentamer assembly) and between pentamers (decamer assembly). Each monomer buries 35.5% of its ASA to form the pentamer assembly. The resulting pentamer is an intertwined structure with the interface predominantly making non-polar contacts (64.8% of non-polar ASA, 245 van der Waals contacts per monomer). Each monomer also makes 6 hydrogen bonds and 1 salt bridge that stabilize the pentamer assembly. Two to three bridging waters per monomer make an additional 6 hydrogen bonds. Each monomer buries 9.2% of its ASA to form the decamer assembly. This interface is also mainly hydrophobic (61.5% of non-polar ∆ASA, 92 van der Waals contacts per monomer). Each pentamer also makes 2 hydrogen bonds that stabilize the decamer assembly. Noteworthy, 10 phosphates make 20 bridging hydrogen bonds stabilizing this interface (the details to be published elsewhere).

**Integrated Mechanism of Decameric BLS Unfolding**—The crystallographic structural analysis, together with the solution...
studies described above, allowed us to postulate a comprehensive model for BLS unfolding (Fig. 6). As shown, the decameric assembly dissociates in two different conditions to stable folded pentamers, with an estimated $\Delta G$ of 90–20 kJ/mol decamer. Thus, BLS would be capable to shift from a decameric to a pentameric quaternary state at acidic pH, conserving the stability of the protein. The effect of the pH on dissociation would be explained by the high density of histidines at the pentamer-pentamer interface (see Fig. 5C). Histidine residues become protonated at pH levels below 6.0, thus producing charge repulsion and loss of contacts resulting in the dissociation of the dimer of pentamers. In contrast, the acidic pH does not disturb the stability of the monomer-monomer interface, because ~35% of the accessible surface area of the monomeric polypeptide is buried in monomer-monomer contacts of hydrophobic nature (Tables II and III). In agreement, GdnHCl-induced pentamer unfolding at neutral and acidic pHs gives approximately the same value of $\Delta G$ (300–330 kJ/mol). This high value of free energy would be the product of the tight and intertwined nature of the monomer-monomer interface, stabilized for a very high number of van der Waals contacts (Table III) resistant to the effect of the low pH.

FIG. 6. Proposed mechanism of dissociation and unfolding of BLS. The dissociation of BLS renders intermediate folded pentamers, without any significant change in the secondary and tertiary structures. Dissociation is induced by 2 M [GdnHCl] at neutral pH; further increase of [GdnHCl] produces the concerted dissociation of the pentamer and unfolding of the 18-kDa monomer. Incubation at pH 5.0 without GdnHCl produces the same pentameric intermediate, with approximately the same thermodynamic stability as judged by GdnHCl denaturation. Thermodynamic estimates of dissociation and unfolding are shown.

| Total contacts | Hydrogen bonds | Salt bridges | Van der Waals contacts |
|----------------|----------------|--------------|------------------------|
| Monomer-monomer interface | 252 | 6 | 1 | 245 |
| Pentamer-pentamer interface | 94 | 2 | 0 | 92 |

| Bridging phosphates | Bridging waters |
|---------------------|-----------------|
| Number | HB | Number | HB |
| 2/3 | 6 | 10 | 20 | 0 | 0 |

Common cellular proteins exhibit only marginal stabilities, with free energies of stabilization of the order of 50 kJ/mol (24). In contrast, quaternary interactions have a dominating role in the stabilization of small oligomeric proteins (25). Unfolding pathways for oligomeric proteins have been shown to vary significantly. Subunit dissociation could occur before or after polypeptide unfolding, or the two reactions could occur simultaneously without significant population of equilibrium intermediates (26). Examples of dissociation to folded monomers followed by monomer unfolding have been described (27). Several examples of dissociation coupled to unfolding have also been described (28–30). The decameric BLS contains elements of both types of mechanisms. The dissociation of the dimer of pentamers and the isolation of a pentameric intermediate population with conserved folded structure, detected around 2.0 M GdnHCl chloride and at acidic pHs, would be the consequence of...
of structural adaptation during quaternary divergence from pentameric to decameric lumazine synthases. In this sense, the continuous undistorted helix 4 of BLS (see introduction) produces a complementary surface between pentamers composed of hydrophobic interactions, in which there is a marked presence of histidine and phenylalanine residues (Fig. 5C). Additionally, phosphate ions help to cement this interface by means of bridging hydrogen bonds. The fact that we have been able to isolate the intermediate folded pentamer in different conditions would allow for detailed mutagenesis and kinetic folding studies of this unusual protein interface. It would allow also for protein engineering procedures in the use of BLS as protein carrier for the development of immunogens (see below). In contrast, the pentamer presents an intertwined structure with each monomer interacting extensively with its adjacent monomers via multiple hydrophobic, hydrogen bond, and salt bridge interactions. As a consequence, dissociation of the pentamer and unfolding of the resulting monomers occurs as a concerted mechanism. Even though the macroscopic methods employed in this study support a two-state model, we cannot rule out the existence of intermediates. Because residues of neighboring monomers form the active site of the enzyme, there are no structural reasons for the existence of folded monomers.

The apparent transition temperature (T_m) of BLS is very high, typical of a protein from a thermophilic organism. Unexpectedly high T_m from other LS were previously described (5). Thermal unfolding of BLS shows an intermediate behavior between pentameric and icosahedric LS, as expected for its pentameric to icosahedric lumazine synthases. In this sense, the lack of an internal riboflavin synthase as in the case of icosahedric enzymes (31) suggests but appear too narrow for the exit of lumazine (32). Although substrate channeling has to be ruled out because of the existence of fluorescent products associated to recombinantly expressed purified BLS (resistant to urea dissociation) support this idea.

BLS folds as a highly stable dimer of pentamers and is a highly immunogenic protein (23, 34, 35). These characteristics resemble that of the B subunits of E. coli heat-labile enterotoxin (EtxB) and cholera toxin (CtxB). Both toxins assemble in vivo into exceptionally stable homopentameric complexes, which maintain their quaternary structure in a range of conditions that would normally be expected to cause protein denaturation (36). These remarkable stability properties, as well as the inherent immunogenicity of EtxB and CtxB pentamers, have prompted considerable interest in their use as vaccine delivery vehicles (36). Noteworthy, BLS is more stable to thermal denaturation than EtxB and CtxB toxins (T_m of 84 and 75 °C, respectively) (37, 38). Thus, BLS is a promising potential carrier for the polymeric delivery of antigens or epitopes. The presence of ten sites of linkage and the natural disordered conformation of its N termini (6) linked to its high stability indicate that BLS is a potential candidate for the development of subunit vaccines.

Acknowledgments—We acknowledge the support of Fundación Instituto Leloir, Dr. Gonzalo Prat Gray for the critical reading of the manuscript, and D. Laplagne for help in the early steps of the work.

REFERENCES

1. Ladenstein, R., Schneider, M., Huber, R., Bartunik, H. D., Wilson, K., Schott, K., and Bacher, A. (1988) J. Mol. Biol. 203, 1045–1070
2. Gerhardt, S., Haase, L., Steinbacher, S., Kaiser, J. T., Cushman, M., Bacher, A., Huber, R., and Fischer, M. (2002) J. Mol. Biol. 318, 1317–1329
3. Bacher, A. (1986) Methods Enzymol. 122, 192–199
4. Ritsert, K., Huber, R., Turk, D., Ladenstein, R., Schmidt-Base, K., and Bacher, A. (1995) J. Mol. Biol. 253, 151–167
5. Zhang, X., Meining, W., Fischler, M., Bacher, A., and Ladenstein, R. (2001) J. Mol. Biol. 310, 1099–1114
6. Braden, B. C., Velikovsky, C. A., Cauerhaff, A. A., Polikarpov, I., and Goldberg, D. J. (2000) J. Mol. Biol. 297, 1031–1036
7. Meining, W., Mortl, S., Fischer, M., Cushman, M., Bacher, A., and Ladenstein, R. (2000) J. Mol. Biol. 299, 181–197
8. Ferreira, M. S., Laplagne, D. A., Frankel, N., Cauerhaff, A. A., Goldberg, F. A., and Chevape, J. (2003) J. Mol. Biol. 10.1016/j.molcel.2003.11.014
9. Goldberg, F. A., Velikovsky, C. A., Baldi, P. C., Mortl, S., Bacher, A., and Fossati, C. A. (1999) J. Mol. Biol. 28, 832–839
10. Greene, R. F., Jr., and Pace, C. N. (1974) J. Biol. Chem. 249, 535–5393
11. Polikarpov, I., Oliva, G., Castellano, E. E., Gurrati, R. C., Arruda, P., Leite, A., and Baldini, G. (1999) Biochim. Biophys. Acta 1397, 103–104
12. Boudker, O., Todd, M. J., and Freire, E. (1997) J. Mol. Biol. 269, 405–425
13. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
14. Polikarpov, I., Moczygemba, C. K., Steede, N. K., Landry, S. J., and Wittung-Stafshede, P. (2000) Protein Sci. 9, 2109–2117
27. Reddy, G. B., Bharadwaj, S., and Surolia, A. (1999) Biochemistry 38, 4464–4470
28. Dams, T., and Jaenicke, R. (1999) Biochemistry 38, 9169–9178
29. Panse, V. G., Swaminathan, C. P., Ahor, J. J., Surolia, A., and Varadarajan, R. (2000) Biochemistry 39, 2362–2369
30. Mok, Y. K., de Prat Gay, G., Butler, P. J., and Bycroft, M. (1996) Protein Sci. 5, 310–319
31. Kis, K., Volk, R., and Bacher, A. (1995) Biochemistry 34, 2883–2892
32. Ladenstein, R., Ritsert, K., Huber, R., Richter, G., and Bacher, A. (1994) Eur. J. Biochem. 223, 1007–1017
33. Kohler, S., Michaux-Charachon, S., Porte, F., Ramuz, M., and Liautard, J. P. (2003) Trends Microbiol. 11, 215–219
34. Goldbaum, F. A., Leoni, J., Wallach, J. C., and Fossati, C. A. (1993) J. Clin. Microbiol. 31, 2141–2145
35. Velkovsky, C. A., Cassataro, J., Giambartolomei, G. H., Goldbaum, F. A., Estean, S., Bowden, R. A., Bruno, L., Fossati, C. A., and Spitz, M. (2002) Infect. Immun. 70, 2507–2511
36. Ruddock, L. W., Coen, J. J., Cheesman, C., Freedman, R. B., and Hirst, T. R. (1996) J. Biol. Chem. 271, 19118–19123
37. Ruddock, L. W., Webb, H. M., Ruston, S. P., Cheesman, C., Freedman, R. B., and Hirst, T. R. (1996) Biochemistry 35, 16069–16076
38. Bhakuni, V., Xie, D., and Freire, E. (1991) Biochemistry 30, 5055–5060