ARTICLE
Journal of Cellular Biochemistry 113:2543–2548 (2012)

Journal of Cellular Biochemistry

Oxygen-Bound Hell's Gate Globin I by Classical Versus LB Nanotemplate Method

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ABSTRACT

X-ray atomic structure of recombinant Hell's gate globin I (HGbI) from Methylacidophilum infernorum was calculated from the X-ray diffraction data of two different types of crystals: obtained by classical hanging drop and by LB nanotemplate method under the same crystallization conditions. After the accurate comparison of crystallographic parameters and electron density maps of two structures they appears to be quite similar, while the quality of the crystals grown by LB nanotemplate method was higher then of those grown by classical method. Indeed, the resolution of the LB crystal structure was 1.65 Å, while classical crystals showed only 3.2 Å resolution. Moreover, the reproducibility of this result in the case of LB crystals was much better—nine crystals from 10 gave the same structural results, while only two of 10 classical crystals were appropriate for the X-ray structure resolution. J. Cell. Biochem. 113: 2543–2548, 2012.

KEY WORDS: HELL'S GATE GLOBIN I; THIN FILM; LANGMUIR BLODGETT

Globins are heme-containing proteins involved in binding and/or transporting oxygen. They belong to a very large and well-studied family that is widely distributed in many organisms [Vinogradov et al., 2007]. Globins are typically composed of eight a-helices, labeled A–H, that fold into a three-over-three a-helical sandwich structure [Holm and Sander, 1993] Helices A, B, C, and E are on the distal side of the heme and helices F, G, and H are on the proximal side. The proximal histidine in the F helix (His F8) binds the heme cofactor to the protein by coordinating to the heme iron's fifth coordinate. Gaseous ligands bind to the iron's sixth coordinate at the opposite side of the heme plane.

Hell's Gate globin I (HGbI), a single-domain protein with 133 residues, was identified from the genome of *Methylacidiphilum infernorum* [Teh et al., 2011], a aerobic acidophilic and thermophilic obligate methanotroph that grows optimally at 60°C and pH 2.0 [Dunfield et al., 2007]. HGbI is structurally homologous to mammalian neuroglobins. Its particular features are: (i) high affinity for the oxygene, (ii) negligible autoxidation in the pH range of 5.2–8.6 and temperature range of 25–50°C, (iii) unique resistance to the extreme acidity. Al this facts makes the HGbI an interesting model in the fundamental structure–functions studies of the globins superfamily, as well as it possible industrial application in the sensors devices [Nicolini, 1997; Green et al., 2009], since hemoglobins exhibit a wide variety of functions, including the reversible binding of oxygen for transport and storage, to cytoprotection against reactive oxygen species, nitric oxide (NO) scavenging, signaling of oxygen-dependent metabolic pathways, and electron transfer [Wajcman et al., 2009].

In this report, we crystallize the HGbI by both classical vapor diffusion and LB nanotemplate method, known to produce more ordered and radiation stable crystals [Pechkova and Nicolini, 2004; Pechkova et al., 2009]. The advantage of use this method is the higher quality of crystal both in terms of X-ray diffraction and radiation stability when use of the high energy X-ray source and focused beans, as third generation synchrotrons, including micro-diffraction beamlines [Pechkova et al., 2004, 2005; Nicolini and

Grant sponsor: MIUR (Ministero dell'Istruzione, Università e Ricerca); Grant number: FIRB RBIN04RXHS; Grant sponsor: Nanobiosensors; Grant number: FIRB ITALNANONET RBPR05JH2P_003 Grant for Funzionamento to Fondazione El.B.A.Nicolini.

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Manuscript Received: 1 March 2012; Manuscript Accepted: 6 March 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 March 2012

DOI 10.1002/jcb.24131 • © 2012 Wiley Periodicals, Inc.

Pechkova, 2004]. The aim of this work is to demonstrate the capability of LB nanocystallography to produce the crystals structures of higher atomic resolution in comparison with classical vapor diffusion technique and confirm the reproducibility of these results.

MATERIALS AND METHODS

HGbI from *Methylacidiphilum Infernorum*, was provided by Maqsudul Alam and Jennifer Saito from University of Hawaii and the Centre for Chemical Biology of University Sains Malaysia, being expressed in *Escherichia coli* and purified accordingly for the final concentration of 10 mg/ml in HGbI in 50 mM Tris–Cl, 200 mM NaCl buffer, pH 7 [Teh et al., 2011] Crystals were grown in Genova University using two different methods, classical vapor diffusion hanging drop method and LB nanotemplate method.

LB NANOTEMPLATE TECHNIQUE

A HGbI thin film was prepared on the water–area interface and compressed to a surface pressure of 20 mN/m by means of a Langmuir–Blodgett trough [Pechkova and Nicolini, 2001, 2004]. A protein monolayer was deposited on the siliconized glass cover slide of 20 mm diameter (Hampton Research) by the Langmuir–Schaeffer method. This highly ordered protein nanotemplate was utilized in a hanging-drop protein crystallization method modification. The drop of protein solution and the precipitant (salt) was placed on the glass slide covered by LB thin film nanotamplate. As in the classical hanging-drop method, the glass slide with the protein template and the drop of protein solution was sealed on the crystallization plate (Limbro plate, Hampton Research) using vacuum grease.

PROTEIN CRYSTALLIZATION

The crystallization conditions were used for the classical vapor diffusion hanging-drop method and LB nanotemplate method. Three microliter protein solution containing 10 mg/ml HGbI in 50 mM Tris–Cl, 200 mM NaCl buffer, pH 7 were mixed with 3 μl of reservoir solution containing 1.4–2 M $(NH_4)_2SO_4$, 0.1 M sodium acetate, pH 4.6 on the siliconized glass slide in the case of classical hanging drop method and on the glass slide covered by LB protein nanofilm in the case of nanotemplate method and were equilibrate over the reservoir (1 ml) at 4°C. The best crystals were found over reservoirs, contained 1.6 M $(NH_4)_2SO_4$ and these crystals were utilized for X-ray analysis.

DATA COLLECTION

All X-ray data were collected at ID 14-4, ESRF, Grenoble, France. Beam with an Energy of 13.200 keV and a wavelength of 0.93928 Å was used with a beam size of 0.1×0.1 mm^2 and a flux per image of $8{,}64 \times 10^{10}$ photon/s/μm^2. All datasets were collected using freshly frozen crystals cryocooled at 50°K using their mother liquor containing in addition 30% of glycerol as cryopretectant. Classical and LB-film based crystals of approximately the same dimensions and shape were used to collect two complete data sets.

Twenty crystals were used during the acquisition session at the ID 14-4, 10 Langmuir–Blodgett technology-based crystals and 10 classical hanging drop vapour diffusion crystals. Nine high-resolution datasets were obtained using 10 LB crystals, and only four datasets of low resolution were obtained using 10 classical crystals. Each dataset was composed of 360 images, which were collected with, and exposure time of 0.3 s and 0.5°. The data were recorded using an ADSC Q315r CCD detector.

Worthy of notice is that contrary to the Single wavelength Anomalous Diffraction (SAD) used by Teh et al. [2011] for solving HGbI protein structure from classical crystals based on Iron atoms of functional group, here we use for both LB and classical the Molecular Replacement with Mosflm/XDS-CCP4.

STRUCTURE DETERMINATION AND REFINEMENT

Data were indexed and integrated with MOSFLM [Leslie and Powell, 2007] for classical crystals and XDS [Kabsch, 2010] for LB. To search spacegroup that fit best with our data, POINTLESS [Evans, 2006] were used. Spacegroup C2221 was recognized, and both crystals were processed in spacegroup C2221. Datasets were then scaled with the program SCALA from the CCP4 software package [Collaborative Computational Project, Number 4, 1994]. LB crystals were processed at a resolution of 1.65 Å, classical were processed at 3.2 Å. With unit cell parameters a = 70.23, b = 126.53, c = 148.24 for LB and a = 70.35, b = 126.51, c = 148.36 for classical one and with $\alpha = \beta = \gamma = 90^\circ$ for both. For the calculation of the number of molecules in the asymmetric unit MATTHEW COEFF of the ccp4 package was used assuming a protein molecular weight of 15,100 kDa.

The crystal structure was solved by molecular replacement using MOLREP [Vagin et al., 1997] and the structure of HGbI Hell's Gate Globin I structure [PDB ID code 3S1I, Teh et al., 2011] Manipulation of files was performed using RASMOL [Sayle and Milner-White, 1995].

TABLE I. Data Collection Statistics for HGbI Classical and LB Crystal at ID14-4 Beamline, ESRF

	Crystallization method	
Parameters	LB	Classic
Beamline	14-4	
Temperature (Kelvin)	50	
Detector	ADSC Quantum Q315r	
Wavelength	0.93928	
Resolution range (Å)	1.65–48.12 (1.65–1.74)	3.20–49.45 (3.20–3.37)
a,b,c (Å)	70.23 126.53 148.24	70.35 126.51 148.36
$\alpha = \beta = \gamma$ (°)	90	90
Completeness (%)	100	99.09
Spacegroup	C 2 2 21	
Mosaicity (°)	0.173	0.98
I/Sigma (%)	17.1 (2.5)	5.6 (4.6)
Cell volume (Å^3)	1317290.57	1320400.81
Rsym	0.067	0.246
Rmrg for individual datasets (%)	0.067 (0.789)	0.246 (0.354)
R factor	0.209	0.227
Free R factor	0.241	0.297
Reflections	75438	10710
Redundancy	7.3	6.4
RMSD on bond length (A)	0.031	0.015
RMSD on angles	2.377	1.560
Water molecules#	466	151

The protein model obtained from molecular replacement stage was then refined against the classical and LB crystals using REFMAC5 [Murshudov et al., 1997, 1999]. Electron density maps were then inspected using COOT, before final refinement step of water molecules were added. Statistics of data collection, processing, and refinement are summarized in Table I. Visual inspection of the two structures were performed using PyMol [The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC; PyMOL, 2012] and a structure alignment between these two structures was performed using Procksi Server/TMAlign [Zhang and Skolnick, 2005; Barthel et al., 2007].

RESULTS AND DISCUSSION

Classical crystals were grown after 1–2 days and the average size of the classical crystals was up to 200 μm while the average size of the LB crystals (Fig. 1) was up to 300 μm, and the LB crystals growth was significantly accelerated (Fig. 2). It confirms the fact that the crystal grown in presence of thin film is larger, than the crystal grown by the classical hinging drop vapour diffusion method, as previously reported [Pechkova and Nicolini, 2001]. The two types of crystals have the same shape but different morphology: the classical ones have irregular surface while LB crystal present clear perfect shape (Fig. 1).

Ten LB and 10 classical crystals were analyzed and diffraction data were collected with several structures obtained for both kinds of crystals. In particular for LB method was possible to resolve nine structures from the 10 possible, while for classical method was possible to reach final solution only for two data sets acquired, due to the lower quality of classical crystals' diffraction data in comparison of those obtained by LB nanotemplate method. The two structures discussed in this article one or classical and one for LB method were obtained from the best diffraction datasets for both type of crystals. Well-defined electron density maps were obtained for both kinds of crystals. The crystallographic parameters are summarized in the Table I.

This represents what we found experimentally with our hands on the 10 crystals taken randomly over the best 100 being grown under the same conditions for each type of preparation (with and without LB). Apparently, the very best result obtainable with the classical preparation under identical conditions appears to yield on other hands a quite better resolution on the very same protein, namely the 1.75 A° resolution (Teh et al., 2011). The diffraction data shown are actually the ones for the very best LB and very best classical in our hands which appear morphologically quite identical in the nylon cryoloop at the ESRF beamline (as shown in Fig. 1b,d). At ESERF indeed we analyzed many smaller crystals of both type which do look both perfect and identical for both classical and LB, while the crystals shown in Figure 1a,c were those of the maximum dimension apparent in Figure 2 and in this case the classical (1b) looks quite worst than LB (1a).

Figure 3 shown the stereo view of the superposition of the Cα-chains of LB and classical HGbI structure, while in the Figure 4

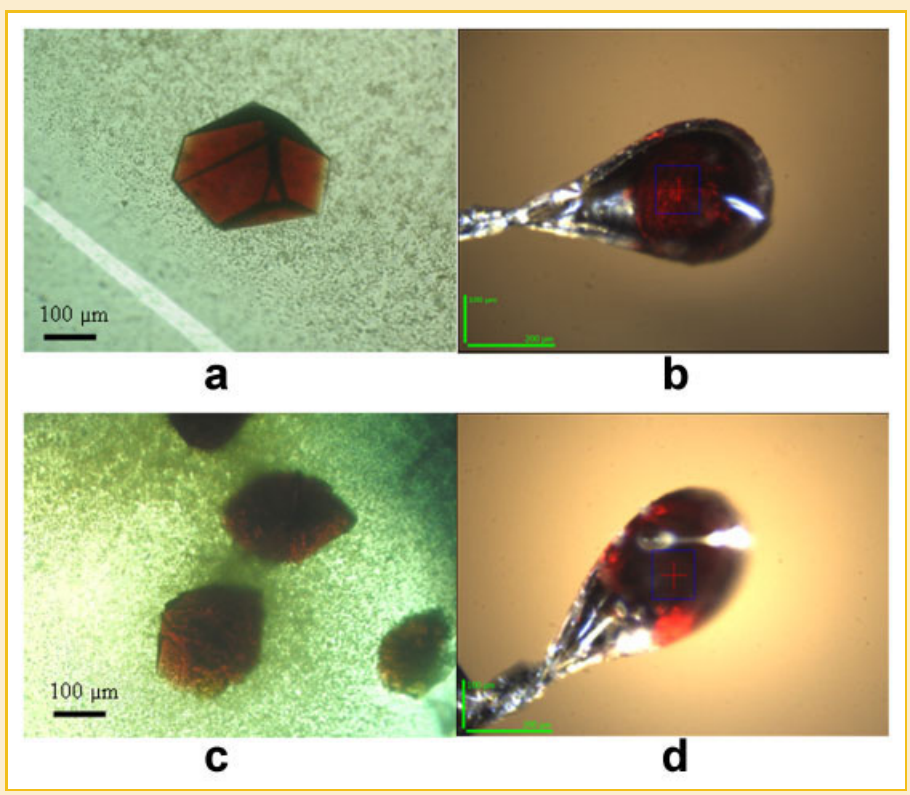

Fig. 1. HGbI crystals grown by LB nanotemplate (a,b) and classical hanging drop method (c,d) under the optic microscope (a,c) and in the nylon cryoloop (b,d) in the ESRF beamline ID14-4; the beam size shown in blue and beam center shown as a red cross.

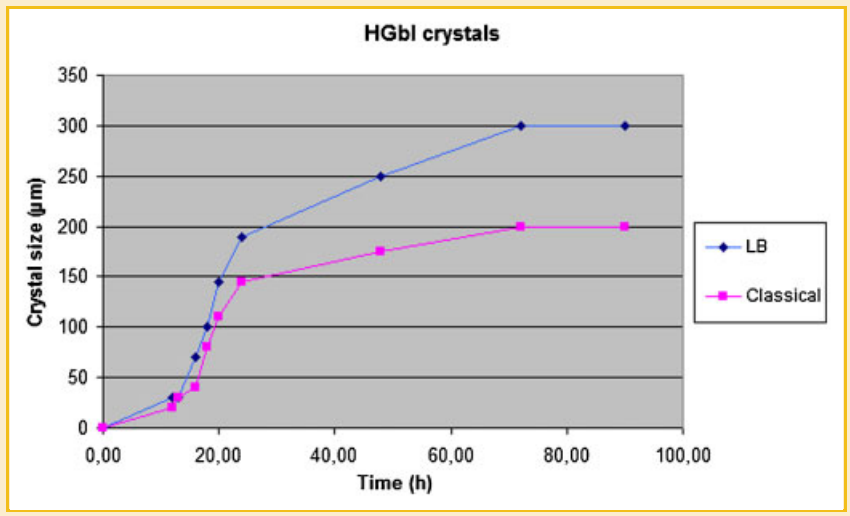

Fig. 2. The acceleration of the HGbI crystal growth by LB nanotemplate method: the classical crystals growth shown in red, the LB crystal growth shown in blue.

are presented the three-dimensional structures obtained for the classical and LB crystals. Anyway, differences were shown in the two kinds of crystals. First of all, LB crystals showed an higher resolution during acquisition step and an higher resolution structure was obtained for this kind of crystals with an average resolution of about 1.8 Å for LB crystals (on nine solved structures above 10 acquired for 10 crystals) versus a 3.3 Å resolution for classical ones (on two solved structures above four acquired for 10 crystals). In particular, in this article, we discussed about an LB crystal solved with a 1.65 Å resolution versus a classical crystal of 3.2 Å resolution.

Solved structures showed differences in the unit-cell parameter, indeed c (148.36 Å in the classical vs. 148.24 Å in the LB crystals) indicates that either packing of the molecules or their conformation (which is less likely considering the quoted RMSD values) may differ between the classical and the LB crystals. A difference in the volume of the unit cell is shown too with a volume of 1317290.57 $Å^3$ for LB and of 1320400.81 $Å^3$ for classical one, showing a difference of about 1% of volume. Besides, comparison of R factors—LB (0.209) and Classical (0.227)—positional errors in coordinates and RMS. Values for bond lengths and bond angles shows that values of these parameters are very similar between the classical and the LB data sets, which rules out insufficient precision of structure determination or different qualities of film- and LB-based crystals as sources of error in comparing the patterns between the LB and the classical crystals.

A visual inspection was performed on the two obtained structures to confirm or deny difference within them and no differences in the secondary structures were observed. A superposing was then performed showing that some differences can be seen in the two structures in terms of atomic coordinates. A Procksi Server/TMAlign [Barthel et al., 2007] task was then performed to compare the 3D structures and quantify differences between them. TMAlign [Zhang and Skolnick, 2005] is an algorithm based on a scoring method called TMScoring used to compare structure with same sequences. A score of 1 means an identical structure. Results are good if scoring is over 0.5, worse results are for a scoring of <0.2. This task was performed using three chains for the two structures and comparing them, that is, every chains of the classical crystal protein with every chain of the LB crystals protein. The results confirm that little differences occur within this two structures as shown in details in Table II with the worse TMScoring of 0.99382, confirming that 3D structures are very similar and no relevant differences appears in

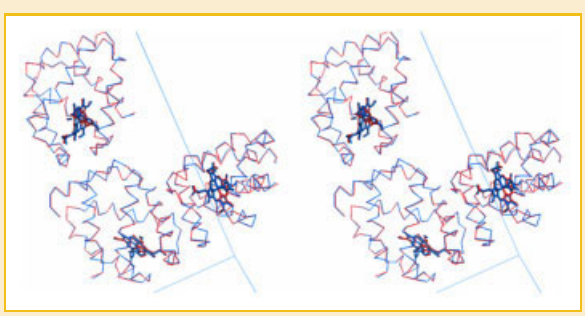

Fig. 3. Stereo view of the superposition of the Cα-chain trace of HGbI protein: classic (blue) and LB (red). Axes are, from leftmost clockwise: y, z, x.

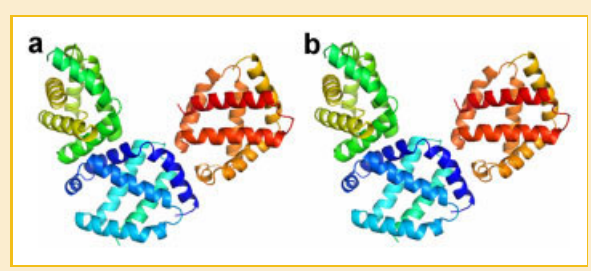

Fig. 4. Three-dimensional structures obtained for the classical (a) and thin film based (b) crystals of HGbI protein.

TABLE II. Procksi Server/TMAlign Results

Structures	ClassAA	classDD	classGG	lbAA	lbDD	LbGG
ClassAA	1.00000 (0)	#	#	#	#	#
ClassDD	0.99240 (0.37)	1.00000 (0)	#	#	#	#
ClassGG	0.99288 (0.36)	0.99095 (0.41)	1.00000 (0)	#	#	#
LbAA	0.99724 (0.22)	0.99133 (0.40)	0.99174 (0.39)	1.00000 (0)	#	#
LbDD	0.99332 (0.35)	0.99609 (0.27)	0.99275 (0.37)	0.99444 (0.32)	1.00000 (0)	#
LbGG	0.99382 (0.34)	0.99182 (0.39)	0.99616 (0.26)	0.99517 (0.30)	0.99546 (0.29)	1.00000 (0)

TMScoring and related RMSD values in brackets.

terms of structures between them. Table II was obtained as output of the TMAlign task performed on ProcksiServer showing TMScoring and RMSD in brackets. Results are reported for every comparison of every chain, with itself and other chains.

CONCLUSION

Hemoglobin consists of four subunits, two α and two β subunit, in which each α and each β forms a dimer, in this way hemoglobin usually form a dimer of α β dimer. Three identical subunits—as shown in Teh et al. [2011] for classical hanging drop crystals of HGbI—were found in the asymmetric unit of both crystals, this is due on the crystal packaging of HGbI. Two subunits are related by a non-crystallographic axes forming a dimer. Second monomer is formed by the third subunit due to a crystallographic twofold axis that occur between two asymmetric units. After the detailed study of electron density map fragmentation and map correlation to the resulting models, LB and classical crystal structures appear to be similar, while the LB crystals are of better quality, diffracting to higher resolution in comparison to the classical crystals. Higher quality of the LB crystal with respect to the classical crystal could result from the difference in the inner water structure, as previously discussed in earlier artcle [Pechkova et al., 2007] and confirmed in more details in a recent characterization of the water distribution at atomic level in several protein systems.

Among 20 obtained crystals under the same crystallization conditions, the resolution of the LB crystal structure was 1.65 Å, while classical crystals showed only 3.2 Å resolution. Moreover, the reproducibility of this result in the case of LB crystals was much better—nine crystals from 10 gave the same structural results, while only two of 10 classical crystals were appropriate for the X-ray structure resolution. Furthermore, it is worth to notice that only Single wavelength Anomalous Diffraction (SAD) was possible to use by Teh et al. [2011] for solving structure based on Iron atoms of functional group of HGbI protein, while Molecular Replacement was been used by us to solve all the LB structures using Mosflm/XDS always with high resolution (in the range [1.7–1.8 Å]) and to solve only few classical structure always with low resolution (about 3.2 Å) and high Rfactor too.

PDB ENTRY

"Oxygen-bound hell's gate globin I by classical hanging drop" RCSB ID code rcsb068558; PDB ID code 3UBV.

"Oxygen-bound hell's gate globin I by LB nanotemplate method" RCSB ID code rcsb068540; PDB ID code 3UBC.

ACKNOWLEDGMENTS

This study was supported by the grant sponsor: MIUR (Ministero dell'Istruzione, Università e Ricerca). Grant contract: Proteomics and Cell Cycle (FIRB RBIN04RXHS), and Nanobiosensors (FIRB ITALNANONET RBPR05JH2P_003), both to Claudio Nicolini University of Genova. Grant contract: Funzionamento to Fondazione El.B.A.Nicolini for 2011 and 2012. We are gratefull to Professor Alam Maqsudul and Dr. Jennifer Saito from University of Hawaii and the Centre for Chemical Biology of University Sains Malaysia for providing the purified HGbI from *Methylacidiphilum Infernorum*. We gratefully acknowledge access to beam time at the ESRF under Radiation Damage BAG.

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