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Dehydration Converts DsbG Crystal Diffraction from Low to High Resolution

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Summary

Diffraction quality crystals are essential for crystallographic studies of protein structure, and the production of poorly diffracting crystals is often regarded as a dead end in the process. Here we show a dramatic improvement of poorly diffracting DsbG crystals allowing high-resolution diffraction data measurement. Before dehydration, the crystals are fragile and the diffraction pattern is streaky, extending to 10 Å resolution. After dehydration, there is a spectacular improvement, with the diffraction pattern extending to 2 Å resolution. This and other recent results show that dehydration is a simple, rapid, and inexpensive approach to convert poor quality crystals into diffraction quality crystals.

Introduction

Despite technical and methodological advances in the field of structural biology, obtaining diffraction quality crystals still remains a major obstacle in protein crystallographic research. Loose packing of molecules and large solvent volume are common problems that result in low-resolution diffraction. Different strategies to overcome this problem have been described in the literature, including the use of postcrystallization treatments, such as annealing after flash freezing and controlled dehydration [1–3]. Crystal dehydration alone or combined with other treatments (cryocooling, annealing, and rehydration) has been reported to improve diffraction resolution by between 0.3 Å and 1.5 Å in most cases [3–13], and by ~3 Å in two reported cases [14, 15]. Here we show that the diffraction resolution of DsbG protein crystals can be improved from 10 Å to 2 Å resolution by crystal dehydration.

DsbG is a member of the Dsb (disulfide bond) family of proteins (DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG), which control the formation of disulfide bonds in the bacterial periplasm [16, 17]. Dsb proteins lack an overall sequence homology, but they all share a CXXC active site motif characteristic of the thioredoxin superfamily [18]. These proteins form two distinct pathways for disulfide formation and rearrangement. The DsbA-DsbB pathway [19–21] rapidly introduces disulfide bonds into target proteins, sometimes resulting in the formation of nonnative disulfide bonds, whereas the DsbC/DsbG-DsbD pathway [22–26] catalyzes the rearrangement of incorrect disulfide bonds, allowing proteins to fold correctly. We are currently investigating the structure of DsbG, a 2 × 25.7 kDa periplasmic homodimer that, like DsbC, shows disulfide bond isomerase activity and which also functions as a molecular chaperone [27, 28]. DsbG is homologous to DsbC (sequence identity 24%) [29], suggesting that it may share a similar fold. We cloned, expressed, and purified DsbG and produced crystals by hanging drop vapor diffusion. However, DsbG crystals produced in this way were fragile and temperature sensitive, and the diffraction pattern was poor. Experimental approaches for improving the quality of the crystals were therefore investigated.

Results

X-ray diffraction of DsbG crystals at room temperature resulted in a diffraction pattern extending to ~10 Å resolution (Table 1A; Figure 1A). The effect of cryocooling by immersing a crystal in a stabilizing solution containing 25% glycerol, and flash cooling the crystal in a nitrogen gas stream at 100 K was investigated. This treatment also resulted in streaky diffraction, extending to ~10 Å resolution (Table 1B; Figure 1B). A gentler cryoprotection scheme, involving stepwise [30] equilibration of crystals in increasing concentrations of glycerol (up to 25% in 5% steps), was also tested. Crystals were equilibrated at each step for ~10 min, with a total transfer time of almost 1 hr. Crystals were then plunged into liquid nitrogen and transferred frozen to the nitrogen gas stream at 100 K. However, crystals treated in this gentler manner also diffracted to ~10 Å (Table 1C; Figure 1C). Temperature annealing [1, 2] was attempted on these crystals by blocking the gas stream for a few seconds and then flash cooling the crystal in the gas stream again. However, this did not improve the quality of the diffraction. Rather, this resulted in the complete absence of a diffraction pattern (not shown).

Improving Diffraction Quality by Dehydration

At this point, our usual protocol is to begin searching for new crystallization conditions to identify a new crystal form or to generate a new construct to produce a different form of the protein. However, because it had been reported that crystals of the DsbG homolog DsbC were dehydrated prior to data collection [29], we decided to test the effect of dehydration on DsbG crystals.

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Table 1. Crystal Treatments and Data Collection Statistics for Native and SeMet DsbG Crystals

DsbG (refer to Figure 1)	A (1a)	B (1b)	C (1c)	D (1d)	E SeMet DsbG
Manipulation temperature (K)	293	293	277	277	277
Dehydration	no	no	no	yes	yes
Cryoprotection	no	yes	yes	yes	yes
Cryoprotectant	–	25% glycerol	5%–25% glycerol[a]	15%–25% glycerol[a]	15%–25% glycerol[a]
Temperature of measurement (K)	293	100	100	100	100
Resolution (Å)	~10	~10	~10	33–2.0	29–2.3
Space group	–	–	*P*2	C2	*C*2
a, *b*, *c* (Å) β (°)	–	–	(72, 118, 219),[c] 92	116.9, 57.2, 85.5, 95	116.8, 57.1, 85.5, 95
Mosaicity (°)	–	–	–	0.46	0.5
Number of observations	–	–	–	116,206	182,790
Number of unique reflections	–	–	–	36,303	24,731
R_{sym}[b] (%)	–	–	–	8.1 (31.3)	11.8 (27.4)
Completeness (%)	–	–	–	95.5 (88.0)	98.2 (97.7)
I/σ (I)	–	–	–	6.4 (2.1)	5.3 (2.5)

[a] Stepwise equilibration of crystals in increasing concentrations of glycerol (up to 25% in 5% steps).
[b] $R_{sym} = \Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle$, where I is the intensity of each individual reflection. Values in parentheses refer to the highest resolution shell.
[c] Approximate values.

Due to the fragility and temperature-sensitive nature of the DsbG crystals (they began to disintegrate/melt after handling at room temperature), crystal handling was performed in the cold room at 4°C rather than in the laboratory at 20°C. This temperature change did not improve the diffraction quality of the crystals (not shown), but the crystals remained intact after handling.

Two dehydration methods were tested. The first was based on the method described in detail by Haebel and coworkers [15] for a DsbC-DsbDα complex which improved diffraction resolution of their crystals from 7 Å to 3.8 Å. This method required the slow addition of a total of eight times the crystallization drop volume (16 μl) of dehydrating solution (containing 30% PEG 4000 and 10% glycerol; see Experimental Procedures for further detail) to the 2 μl drop containing the DsbG crystal. The drop was then equilibrated against air for a period of 20 min. However, DsbG crystals treated in this way began to crack after a few minutes and were not suitable for X-ray diffraction studies. The second dehydration method was more gentle, involving the transfer of a crystal from the crystallization drop into a 5 μl hanging drop of the same dehydrating solution, which was then equilibrated against a 1 ml volume of dehydrating solu-

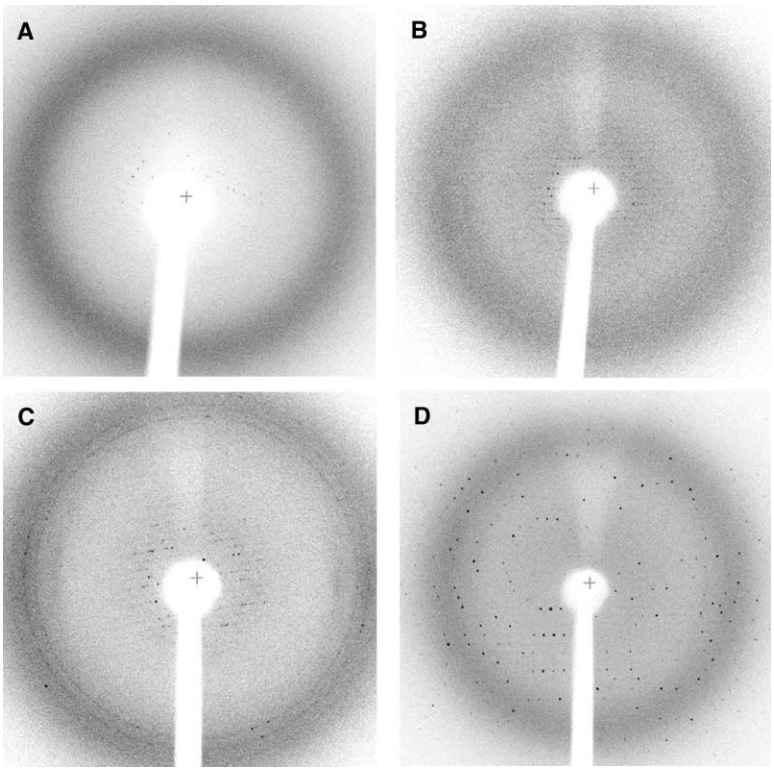

Figure 1. Comparison of X-Ray Diffraction Patterns of DsbG Crystals under Different Conditions

(A) Diffraction image of DsbG crystals at room temperature (resolution ~10 Å).

(B) Diffraction image of DsbG crystals cryoprotected in 25% PEG 4000, 0.1 M sodium citrate (pH 3.75), 0.2 M ammonium sulfate, 25% glycerol and measured at 100 K (resolution ~10 Å).

(C) Diffraction image of DsbG crystals cryoprotected in a stepwise manner by equilibrating the crystals in solutions containing increasing concentrations of glycerol (5%–25%, in 5% increments; resolution ~10 Å, other reflections correspond to an ice ring).

(D) X-ray diffraction pattern after dehydration. Crystals diffract to ~2 Å and have sharply defined spots. For data collection, the dehydrated crystals of DsbG were cryoprotected in a stepwise manner (see text).

tion overnight at 4°C. Crystals of DsbG dehydrated in this way were much more robust than untreated crystals.

Prior to diffraction data measurement, dehydrated crystals were stepwise cryoprotected by soaking in solutions containing increasing concentrations of glycerol. The crystals were equilibrated for 10 min at each step from 15% to 25% glycerol and were then frozen by plunging into liquid nitrogen. The crystals were then transferred frozen to the gaseous nitrogen stream (100 K).

Dehydrated crystals of DsbG exhibited a dramatic improvement in diffraction quality, with the pattern extending to high resolution, and with no evidence of the former streakiness in spots (Table 1D; Figure 1D). A dehydrated DsbG crystal was used to measure 2 Å resolution diffraction data on our laboratory X-ray diffraction equipment (Table 1D). Although it is possible that the improvement in resolution could be progressive, we did not attempt serial transfer into increasing concentrations of precipitant because the one-step procedure worked so well. However, this may be necessary for some crystals [3]. Also, we did not observe intermediate improvements in diffraction resolution nor did we observe further improvement after longer incubation periods. The solvent content based on two subunits (one homodimer) per asymmetric unit was 53% after dehydration, which is within the usual range for proteins (27%–65%) [31]. This contrasts markedly with the estimated solvent content of ~90% for crystals prior to dehydration (an approximate figure due to the difficulty in measuring accurate cell constants from 10 Å data).

The presence of glycerol in the overnight dehydrating solution is not essential for the improvement in diffraction. The same dehydration procedure described above was used excluding glycerol from the dehydration solution. After overnight incubation, the crystal was stepwise cryoprotected with 0% to 25% glycerol. Crystals dehydrated in this way also diffract to ~2 Å resolution (data not shown). However, inclusion of 10% glycerol in the dehydrating solution is convenient because it removes a few steps in the tedious stepwise cryoprotection procedure. Dehydration with 30% PEG 4000 and 25% glycerol was not investigated, but this may be feasible and would circumvent the need for stepwise cryoprotection.

Dehydration of SeMet Crystals

Dehydration is also applicable to selenomethionine-labeled DsbG (SeMet DsbG). Crystals of SeMet DsbG grew under the same conditions as native DsbG crystals. Furthermore, the dehydration procedure used for native crystals was reproduced successfully for SeMet DsbG crystals, allowing measurement of diffraction data to 2.3 Å resolution on our laboratory rotating anode X-ray source (Table 1E).

Discussion

It is well known that water plays an essential role in maintaining the structure of proteins, not only in solution but also in crystalline form [32, 33]. Since very early days, crystallographers have studied in detail the effect of water removal on protein crystals and have investigated the solvent content in and around protein crystals in order to improve their diffraction quality and resolution limit [34–42].

In this paper, we show the most dramatic improvement in diffraction resolution so far described after crystal dehydration. The gentle dehydration method used here resulted in a spectacular improvement of the diffraction resolution of DsbG crystals, from 10 Å to 2 Å resolution as measured on our laboratory X-ray equipment. The diffraction resolution is likely to be even further improved at a synchrotron radiation source. Cryocooling alone or crystal annealing alone had no beneficial effect on the diffraction quality of DsbG crystals.

Crystal dehydration is applicable to protein crystals other than DsbG, with several other cases having been previously reported in the literature (Table 2). Thus, Schick and Jurnak demonstrated that the diffraction resolution of crystals of the guanidine nucleotide exchange factor complex grown from PEG 4000 improved from 4 Å to 2.7 Å resolution by addition of high molecular weight PEG to single crystals, which was accompanied by a decrease in solvent content (Table 2). They obtained a further improvement in resolution by lowering the temperature of data collection (2.5 Å resolution at 250 K) [3]. These observations were extended by Kawashima and coworkers who, working on the same crystal system, found that it was possible to interconvert between high- and low-diffraction crystal forms by a dehydration/hydration process [4].

In another example, Cramer and Muller reported that the anisotropic diffraction exhibited by NF-κB P52:DNA cocrystals (2.4 Å resolution in two directions and 3.5 Å resolution in the third) could be corrected by crystal dehydration, yielding crystals that diffracted beyond 2 Å resolution (Table 2) [5]. Also, crystal dehydration of HIV-1 reverse transcriptase (RT) in complex with inhibitors resulted in a variety of unit cells, the best ordered of which showed an improvement of diffraction resolution from 3.7 Å to 2.2 Å resolution (Table 2) [6].

Tong and coworkers reported another interesting postcrystallization treatment which involved the transfer of human cytomegalovirus (HCMV) protease crystals to an artificial mother liquor containing higher PEG and salt concentrations which significantly improved the diffraction quality (from 3 Å to 2.5 Å resolution in house, and to 2 Å resolution at a synchrotron; Table 2) [7]. Other reports show that dehydration in combination with other factors improves the diffraction quality and resolution of many protein crystals (Table 2). For example, Yang and coworkers showed an improvement of diffraction of *Rv2002* gene product crystals upon annealing and dehydration [8]. The method they used was to flash freeze a crystal grown from PEG 3000 using 10% 2-methyl-2,4-pentanediol as cryoprotectant. The crystal was then removed from the nitrogen-gas stream, placed into a drop of the PEG/cryoprotectant solution, and allowed to air dry. Flash cooling of the annealed and dehydrated crystal improved the diffraction resolution from 2.1 Å to 1.8 Å. This group also showed that the limiting resolution and diffraction quality of peptide deformylase crystals could be improved (from 2 Å to 1.85 Å resolution) by the same crystal annealing/dehydration procedure (Table 2) [9].

Table 2. Dehydration of Protein Crystals and Effect on Solvent Content and Diffraction Resolution

Protein crystal	Ref.	Crystal precipitant[a]	Dehydrating agent	Dehydration treatment	Solvent content[b] before	Solvent content[b] after	Resolution before	Resolution after
DsbG	-	20% PEG 4K	30% PEG 4K	Transfer to drop of dehydr soln, hang over reservoir of dehydr soln, 4°C, 12 hr	~90%	53%	~10 Å[c]	2.0 Å[c]
EF-Tu-Ts	3	20% PEG 4K	28%–40%, var PEGs	Serial transfer, 5 min each	61%	55%	4.0 Å[c]	2.7 Å[c]
NF-κB P52:DNA	5	4%–6% PEG 4K	Ppt + 30% PEG 400 + heavy atom	Serial transfer into increasing PEG 400	52%	49%	3.5 Å[d]	2.0 Å[d]
HIV-RT:inhibitor	6	6% PEG 3.4K	46% PEG 3.4K	Serial transfer, 5% increments, 3 days	56%	48%	3.7 Å[c]	2.2 Å[c]
HCMV protease	7	16% PEG 4K	30% PEG 4K + Na_2SO_4	Serial increase in reservoir conc, 3–5 days	58%	56%	3.0 Å[c]	2.5 Å[c] (2.0 Å[d])
Rv2002 gene product	8	20% PEG 3K	Ppt + 10% MPD	Anneal + air dehydrate, 5 hr	NR	35%	2.1 Å[d]	1.8 Å[d]
Peptide deformylase	9	12% PEG 4K	20% PEG 4K/10% PEG 400	Anneal + air dehydrate, 30 min	NR	50%	2.0 Å[d]	1.85 Å[d]
FAD-indep ALS	10	6%–8% PEG 8K + 6%–9% EG	Ppt + 30% PEG 600	Hang over same dehydr soln, 12 hr + cryocool	NR	52%	2.9 Å[c]	2.6 Å[c]
DsbC-DsbDα	15	25% MPEG 5K + 5% glycerol	40% MPEG 5K + 10% glycerol	Air dehydrate 30 min + cryocool	55%	41%	7.0 Å[c]	3.8 Å[c] (2.3 Å[d])
PDH	14	6% PEG 3K	Ppt + 35% glycerol	Air dehydrate for 28 months, rehydrate in same soln, cryocool	NR	73%	7.0 Å[d]	4.2 Å[d]
Monoclinic lysozyme	11	3% $NaNO_3$	Satd K_2CrO_4 solution	Seal crystal in capillary, add plug of dehydr soln, for 15–20 hr	33%	22%	2.5 Å[c]	1.75 Å[c]
Tetragonal lysozyme	12	0.48–0.75 M NaCl	Satd salt solutions	Seal crystal in capillary, add plug of dehydr soln, for days to weeks	NR	NR	1.6 Å[d]	3.7 Å[d]
MTCP-1	13	1.5 M $(NH_4)_2SO_4$	2.0 M $(NH_4)_2SO_4$	Soaked for 1–5 months	41%	37%	3.0 Å[c]	2.0 Å[c]

[a]Crystal precipitant information does not include details of buffers and other additives used in crystallization.
[b]Solvent content was not always reported by authors, but in most cases it could be calculated from information provided in the text of the paper.
[c]X-ray diffraction resolution on a rotating anode source.
[d]X-ray diffraction resolution at a synchrotron source.
Dehydr soln, dehydrating solution; MPD, 2-methyl-2,4-pentanediol; MPEG, PEG monomethylether; NR, not reported; PEG, polyethylene glycol; ppt, precipitant; satd, saturated; var, various.

Recently, Pang and coworkers reported that crystals of FAD-independent acetolactate synthase improved from 2.9 Å to 2.6 Å resolution by a combination of dehydration and cryocooling [10]. In this case, crystals were grown from PEG 8000 and ethylene glycol, and dehydrated by equilibration against a reservoir solution that included 30% PEG 600 (cryoprotectant and dehydrating agent). Also, Haebel and coworkers showed that dehydration by air equilibration combined with cryocooling improved the diffraction quality of crystals of a DsbC-DsbDα complex from 7 Å to 3.8 Å resolution on a rotating anode X-ray generator and to 2.3 Å resolution at a synchrotron [15].

Diffraction to higher resolution upon rehydration of dehydrated crystals has also been reported. Thus, *Enterococcus faecalis* pyruvate dehydrogenase (PDH) core crystals initially diffracted to 7 Å resolution. These crystals were dehydrated for a period of 28 months and then rehydrated. This treatment extended the diffraction resolution to 4.2 Å resolution (Table 2) [14].

The protein crystals described above were grown from PEG precipitant (Table 2). However, dehydration to improve diffraction is not limited to such crystals. Madhusudan and coworkers found that reducing the solvent content from 33 to 22% in monoclinic lysozyme crystals grown from salt improved the diffraction resolution from 2.5 Å to 1.75 Å resolution (Table 2) [11]. Interestingly, for tetragonal lysozyme crystals grown from salt, the diffraction pattern deteriorates from 1.6 Å to 3.7 Å after dehydration (Table 2) [12]. Another example is that of MTCP-1 protein crystals, which grow from ammonium sulfate, where the diffraction resolution was improved from 3 Å to 2 Å after postcrystallization soaking in 2 M ammonium sulfate for 1–5 months (Table 2) [13].

Dehydration of DsbG crystals is the most striking example so far reported of dehydration improving the diffraction quality of crystals, in that the limiting resolution improves from 10 Å to 2 Å. The solvent content of these crystals is reduced by almost 40% by dehydration, which is also the most dramatic change so far reported (generally $\sim$2%–10%; Table 2). Clearly, in this case, the poor diffraction quality of DsbG crystals is associated with the very high solvent content ($\sim$90%). The high solvent content also makes the crystals fragile and difficult to handle. After dehydration the crystals are very robust and diffract to high resolution.

We did not expect such a spectacular improvement in crystal quality upon dehydration of DsbG crystals. Previous dehydration studies had been performed on crystals that diffracted reasonably well (2–4 Å) and the incremental improvement in diffraction resolution was relatively small. Indeed, dehydration was a last resort for DsbG crystals and was only considered because of the recently published work of Haebel et al. [15] on DsbC showing improvement from 7 Å to 3.8 Å resolution. Our finding that crystal dehydration can convert very poor quality crystals (10 Å) to high-resolution data quality crystals (2 Å) is of considerable importance. It means that production of very poor quality crystals may not necessarily require the experimenter to return to time-consuming steps such as cloning to produce a new construct or crystallization to produce a different crystal form. The dehydration postcrystallization treatment we describe here is quick and simple and has the potential to convert an otherwise useless protein crystal into a diffraction quality crystal. We anticipate that, as with crystal annealing, crystal dehydration may not work in all cases but will provide a spectacular improvement in a portion of cases. Furthermore, we expect that crystal dehydration is most likely to improve diffraction in crystals where poor diffraction is due to high solvent content.

Experimental Procedures

Protein Production

To prepare DsbG for crystallization, the *dsbG* gene was cloned into a pET42b vector (Novagen) to produce a C-terminally octa-histidine-tagged DsbG. The plasmid *pET42b::dsbG* was transformed into the *Escherichia coli* strain BL21(DE3). An overnight culture was used to inoculate 1 L of LB medium containing 50 μg ml^{-1} of kanamycin. One milliliter of 1 M isopropyl-D-thiogalactopyranoside (IPTG) was added to the cells at mid-log phase ($OD_{600\ nm}$ $\sim$0.5) to induce protein expression. The cells were harvested by centrifugation (6,000 $\times$ g, 20 min, 277 K) 3 hr after induction, and the periplasmic fraction was obtained by cold osmotic shock. Briefly, the cell pellet was resuspended in 0.2 M Tris buffer (pH 8) with 20% sucrose. After 10 min of stirring, cells were centrifuged at 6,000 $\times$ g for 15 min and the pellet was resuspended in 10 mM Tris buffer (pH 8) by vigorous agitation for 10 min at 277 K. The periplasmic fraction was then separated from protoplasts by centrifugation (16,000 $\times$ g for 30 min at 277 K).

Histidine-tagged DsbG was purified using cobalt-chelate chromatography following standard protocols (Clontech). DsbG was oxidized by addition of 1.7 mM copper(II)[1,10-phenanthroline] and then dialyzed overnight against 20 mM 4-(2-hydroxyethyl)-1-piperazine-N-2-ethanesulfonic acid (HEPES; pH 6.7), 50 mM NaCl. DsbG was further purified using gel filtration Sephacryl S-200 (Pharmacia) chromatography followed by ion exchange chromatography (Bio-Rad Econo-Pac High-S cartridge). The protein eluted from an S-200 gel filtration column between albumin (67 kDa) and ovalbumin (43 kDa) standards, indicating that the homodimeric form of DsbG (54 kDa) had been purified. Fractions containing DsbG were pooled and concentrated to 15 mg ml^{-1} in 20 mM HEPES buffer (pH 6.7), 50 mM NaCl.

Selenomethionine (SeMet) DsbG was expressed from BL21(DE3) strain in minimal medium containing seleno-DL-methionine at a concentration of 50 μg ml^{-1} using methods similar to those described for SeMet CcmG (DsbE) [43]. SeMet DsbG was purified following the same procedures described above for native DsbG, and mass spectrometry of the purified protein, using a SCIEX QSTAR Pulsar QqTOF, confirmed incorporation of selenium.

Crystallization and Diffraction Data Measurement

Crystals of DsbG were grown using the hanging drop vapor diffusion method in 24-well plates at 293 K. Initial trials were carried out using commercial crystallization screens (Hampton Research Crystal Screen 1 and 2), which failed to produce crystals. However, solutions containing polyethylene glycol (PEG) 4000 and PEG 8000 produced promising results (granular precipitate). Further trials were carried out using the commercial Jena Bioscience Crystal Screen kits. We selected screens containing PEG 400, 4000, 6000, 8000, and 20,000 for trials. Microcrystals appeared after 3–4 days in 25% PEG 4000, 0.1 M sodium acetate (pH 4.6), 0.2 M ammonium acetate and in 16% PEG 6000, 0.01 M sodium citrate. After several rounds of optimization, which included changing variables such as temperature, pH, protein concentration, and PEG 4000 and ammonium sulfate concentration, twinned and plate-shaped crystals were obtained. Additive screens and detergent screens from Hampton Research were used to gauge their effect on crystal quality. Single crystals of DsbG grew from 20% PEG 4000, 0.1 M sodium citrate (pH 3.75), 0.2 M ammonium sulfate. Crystals also grew from these conditions in the presence of detergents such as CYMAL 6 and additives like DMSO. The stabilizing solution used for these crystals has 5% more of the precipitant (25% PEG 4000), and the dehydrating solution

contains 10% more of the precipitant (30% PEG 4000) and, in some cases, 10% glycerol, though this is not essential (see text).

Diffraction data were measured using a Rigaku RU-H2R X-ray generator and an R-AXIS IV^{++} area detector with Osmic mirrors. A copper rotating anode was used to produce X-rays of wavelength 1.542 Å. The crystal to detector distance was 150 mm and the 2θ angle was 0°. The cooled nitrogen stream was produced using a CryoIndustries CryoCool-LN2 (model NFC-1259-XRD). Data were processed and scaled using CrystalClear 1.3 (an integrated program for the collection and processing of area detector data, Rigaku Corporation, 1997–2002).

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