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Novel versatile cryoprotectants for heavy-atom derivatization of protein crystals

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Determination of a suitable cryoprotectant agent condition is one of the time-consuming processes in protein X-ray crystallography. The adaptability of two novel versatile cryoprotectants (oil- and water-based types) has been evaluated by cryodiffraction experiments using crystals of five different proteins. The results suggest that complementary use of both oil- and water-based cryoprotectants enables the successful cryoprotection of protein crystals. Since the versatile cryoprotectants do not contain crystallization solution, rapid and efficient preparation of heavy-atom derivatives is possible with these cryoprotectants in the presence of heavy-atom reagents. Four heavy-atom reagents [K_2PtCl_4, $HgCl_2$, $KAu(CN)_2$ and $K_2Pt(CN)_4$] were examined and could be stored with sufficient derivatizing capabilities for over one month in the versatile cryoprotectants.

1. Introduction

X-ray crystallography is one of the most powerful techniques for the three-dimensional structure determination of proteins at atomic resolution. Recently, high-throughput and automated methodologies implemented by structural genomics initiatives have accelerated the process of acquiring structural information. The RIKEN Structural Genomics Initiative (RSGI; http://www.rsgi.riken.go.jp/) is one of the major structural genomics projects in Japan (Yokoyama *et al.*, 2000). The Advanced Protein Crystallography Research Group of RIKEN Harima Institute promotes high-throughput X-ray crystallography using synchrotron radiation at SPring-8, Japan. In the RIKEN Harima Institute, several systems for rapid structure determination have recently been developed, including the automated crystallization robot TERA (Sugahara & Miyano, 2002), the SPring-8 Precise Automatic Cryo-sample Exchanger (SPACE) at beamline BL26B2 (Ueno *et al.*, 2004) and the heavy-atom searching program *HATODAS* (Sugahara *et al.*, 2005; http://hatodas.harima.riken.jp/). However, the current experimental techniques used in protein crystallography are not necessarily adaptable to structural genomics, which handles a huge number of protein crystals. In particular, versatile reagents for cryoprotection and heavy-atom derivatization are desirable.

Cryoprotection methods for protein crystals were developed in order to reduce radiation damage during data collection (Low *et al.*, 1966; Haas & Rossmann, 1970; Petsko, 1975; Rodgers, 1997; Garman & Schneider, 1997). The first step of cryoprotection is to find a cryoprotectant agent for the protein crystal of interest that is suitable to obtain a vitreous phase at cryogenic temperature by flash-cooling. In the conventional procedure, the crystal is soaked in a stabilizing

solution containing reagents which prevent ice formation, such as glycerol (Haas, 1968), ethylene glycol, 2-methyl-2,4-pentanediol (MPD), ethanol, 2-propanol (Petsko, 1975), sucrose (Haas & Rossmann, 1970) and lithium salts (Rubinson *et al.*, 2000). The suitable cryoprotectant is optimized for high-resolution diffraction and low mosaicity after flash-cooling (Mitchell & Garman, 1994). Unoptimized cryoprotectant often results in cracking or dissolution of the crystal. In general, the composition of the suitable cryoprotectant depends upon the physical or chemical properties of the crystal, including solvent content and crystallization conditions. Screening for a suitable cryoprotectant is laborious and time-consuming if a number of crystals obtained under different conditions need to be examined. Therefore, a versatile cryoprotectant suitable for a variety of crystals is desirable. Paratone-N, an oil with high viscosity, has been reported as a versatile cryoprotectant (Hope, 1988). However, crystals flash-cooled with Paratone-N are poorly visible on cryoloops, hampering the alignment of the crystals in the X-ray beam. This is because of the difficulty in removing the excess Paratone-N owing to its high viscosity. Furthermore, application of Paratone-N to crystals with a high solvent content or in combination with crystallization reagents with a low cryoprotection efficiency leads to the formation of an ice ring. This is because oils do not enter the crystals but only provide a barrier against dehydration, in contrast to conventional cryoprotectants which penetrate the lattice. In such cases, it is necessary to remove the remaining mother liquor on the crystal surface. However, this treatment often damages the crystal and leads to an increase in crystal mosaicity. Thus, success in cryoprotection with Paratone-N depends greatly on the dexterity of the person handling the crystals. In order to overcome this problem, the use of Paratone-N with 10% glycerol has been recommended (Kwong & Liu, 1999). Using this combination, formation of ice is greatly reduced. However, the problem of high viscosity persists. Dry liquid paraffin has been suggested as another versatile cryoprotectant (Riboldi-Tunnicliffe & Hilgenfeld, 1999). Application of dry paraffin can solve the visibility/ice-ring problem of Paratone-N-based cryoprotectants. However, dried liquid paraffin is not commercially available and the soaking time should be optimized to obtain acceptable results. Furthermore, oil-based cryoprotectants including Paratone-N and dry liquid paraffin are not always generally useful because a substantial number of protein crystals are damaged by being soaked in them. In such cases, water-based cryoprotectant may be helpful. Therefore, further development of versatile cryoprotectants is needed for rapid crystal screening in structural genomics.

Preparation of heavy-atom derivatives is also a time-consuming process in protein crystallography. The conventional method of preparation of derivatives by soaking with heavy-atom reagents remains an effective method for the determination of crystal structures. However, the preparation and screening of heavy-atom derivatives and cryoprotectants are laborious and time-consuming and are based on 'trial-and-error' procedures. In conventional heavy-atom soaking experiments, the soaking solutions are typically prepared by dissolving heavy-atom reagents in the precipitant solution used for crystallization. It is necessary to prepare the soaking solutions individually for crystals obtained under different conditions. Furthermore, owing to the high reactivity of heavy-atom reagents, the soaking solutions should be freshly prepared. Therefore, more efficient methods for the preparation of heavy-atom derivatives are desirable for structural genomics efforts.

Here, we describe two novel oil- and water-based cryoprotectants suitable for crystals obtained under a range of crystallization conditions. This investigation of versatile cryoprotectants will be useful for efficient cryoprotection of protein crystals in the structural genomics pipeline. We also describe rapid and efficient methods for the preparation of heavy-atom derivative crystals using these novel cryoprotectants in the presence of heavy-atom reagents.

2. Materials and methods

2.1. Crystallization and data collection

Six proteins from *Thermus thermophilus* HB8 (ID00367, ID00126 and ID00069) and *Pyrococcus horikoshii* OT3 (ID10017, ID11731 and ID10754) were crystallized using the oil-microbatch method implemented in the TERA automatic crystallization system (Sugahara & Miyano, 2002). The crystals were grown at 295 K from mother liquor prepared by mixing 0.5 µl protein solution with an equal volume of precipitant solution: 2.75 *M* sodium chloride, 0.1 *M* citrate buffer pH 4.8 for ID00367; 2.75 *M* sodium chloride, 0.1 *M* acetate buffer pH 4.4 for ID10017; 0.5 *M* lithium sulfate, 0.1 *M* citrate buffer pH 4.6 for ID00126; 27.5%(*w*/*v*) PEG 4000, 0.1 *M* HEPES buffer pH 7.4 for ID11731; 27.5%(*w*/*v*) PEG 4000, 0.1 *M* Tris buffer pH 8.2 for ID00069; 3.85 *M* sodium formate, 0.1 *M* acetate buffer pH 5.4 for ID10754. The crystals grew to typical maximum dimensions of 0.20 × 0.16 × 0.07 mm for ID00367, 0.15 × 0.10 × 0.05 mm for ID10017, 0.18 × 0.14 × 0.07 mm for ID00126, 0.20 × 0.05 × 0.05 mm for ID11731, 0.10 × 0.04 × 0.03 mm for ID00069 and 0.15 × 0.10 × 0.10 mm for ID10754.

Crystals were mounted in cryoloops and flash-cooled by placing in a cryostat nitrogen stream at 100 K. Diffraction experiments for cryoprotection were performed using an in-house diffractometer. Two diffraction images with 1° oscillation angle from two orthogonal crystal orientations were collected using in-house Cu $K\alpha$ radiation with a Rigaku R-AXIS-IV image-plate detector. Crystal mosaicity was evaluated using the program *HKL*2000 (Otwinowski & Minor, 1997). The resolution limit was judged by a σ cutoff at $I/\sigma(I) = 5$. The average values of the resolution limit and mosaicity from three crystals are presented. Complete diffraction data sets for the derivatization experiments were collected using in-house Cu $K\alpha$ radiation with Rigaku R-AXIS IV and VII image-plate detectors as well as the beamline SPACE facility (Ueno *et al.*, 2004) with a Rigaku Jupiter CCD detector at BL26B2 of SPring-8 (Hyogo, Japan). All data were processed with *HKL*2000.

Table 1
Results of experiments for the preparation of heavy-atom derivatives.

Protein ID	Heavy atom	Glycerol concentration (%)	Heavy-atom concentration (m*M*)	Soaking time (s)	Derivatization	Age of heavy-atom reagent solution (d)	Crystal dimensions (mm)
Oil-based cryoprotectant							
00367	K_2PtCl_4	5	5% of 10	240	(Ice formation)	0	0.20 × 0.15 × 0.07
		10	5% of 10	240	Success	0	0.20 × 0.15 × 0.07
		10	5% of 10	180	Success	6	0.15 × 0.15 × 0.10
		10	5% of 10	240	Failure	20	0.18 × 0.15 × 0.08
		9	10% of 50	240	Success	21	0.20 × 0.16 × 0.07
		9	10% of 50	240	Success	35	0.20 × 0.15 × 0.08
Water-based cryoprotectant							
00367	K_2PtCl_4	45	10	300	Success	0	0.25 × 0.25 × 0.04
		45	10	120	Success	15	0.15 × 0.15 × 0.08
		45	10	120	Success	28	0.15 × 0.12 × 0.06
		45	10	120	Success	57	0.15 × 0.15 × 0.06
		45	10	120	Success	66	0.15 × 0.15 × 0.05
		45	10	300	Success	180	0.25 × 0.15 × 0.08
		45	1	300	Failure	1	0.25 × 0.15 × 0.08
		45	5	120	Success	2	0.15 × 0.13 × 0.07
00126	$HgCl_2$	45	10	180	Success	0	0.18 × 0.15 × 0.07
		45	10	180	Success	12	0.20 × 0.10 × 0.05
		45	10	180	Success	39	0.20 × 0.10 × 0.05
		45	10	180	Success	53	0.18 × 0.10 × 0.10
	$KAu(CN)_2$	45	10	180	Success	0	0.20 × 0.10 × 0.08
		45	10	180	Success	15	0.18 × 0.14 × 0.07
		45	10	180	Success	35	0.15 × 0.15 × 0.08
		45	10	180	Success	48	0.15 × 0.15 × 0.10
10754	$K_2Pt(CN)_4$	45	10	120	Success	33	0.15 × 0.10 × 0.10

2.2. Preparation and usage of cryoprotectants

Compositions of the oil- and water-based cryoprotectants were established through cryoprotection experiments using crystals from five proteins (ID00367, ID00126 ID00069, ID10017 and ID11731). For the oil-based cryoprotectant, Paratone-N with 10% glycerol (Kwong & Liu, 1999) was used as the starting point. Paraffin oil was added to reduce the viscosity of the cryoprotectant, which can improve the visibility and handling of crystals. For the water-based cryoprotectant, hyaluronic acid was chosen as a commercially available highly viscous hydrophilic polymer that may be useful to prevent quick diffusion of cryoprotectant into protein crystals and to avoid osmotic shock upon soaking (Garman, 1999). The composition was then optimized experimentally. To establish the composition of the water-based cryoprotectant, hyaluronic acid concentrations of 0, 0.02, 0.025, 0.03, 0.13, 0.25, 0.33, 0.5 and 1.0%(*w*/*w*) and glycerol concentrations of 0, 30, 40, 45, 48 and 60%(*w*/*w*) were examined. Firstly, 0.5 and 1.0% aqueous solutions of hyaluronic acid without glycerol were tried. However, ice-ring formation was observed. In the case of 40 and 60% aqueous solutions of glycerol without hyaluronic acid, the crystals were damaged by the cryoptotectants. Thus, the cryoprotectant from the combinations of 0.02, 0.13 and 0.33% hyaluronic acid and 30, 40 and 48% glycerol were examined. Further optimization of the cryoprotectant was performed using intermediate concentrations of the components. The final compositions of the oil- and water-based cryoprotectants were as follows. The oil-based cryoprotectant consisted of 66.5%(*w*/*w*) Paratone-N, 28.5%(*w*/*w*) paraffin oil and 5%(*w*/*w*) glycerol. The water-based cryoprotectant consisted of 0.13%(*w*/*v*) hyaluronic acid and 45%(*w*/*v*) glycerol in aqueous solution.

The oil-based cryoprotectant was degassed by sonication for 15 min. 50–100 mg of the cryoprotectant was put on a glass plate and a crystal was soaked in the protectant for 10 s. Owing to the high viscosity of Paratone-N, the remaining mother liquor around the crystal was removed by moving a cryoloop close to the crystal, which could whisk away the excess mother liquor without touching the crystal. Since the crystallization solution will freeze on cooling, it was important to remove as much of it as possible from the surface of the protein crystal in order to avoid mechanical damage to the crystal from ice formation and to avoid powder diffraction from ice crystals. The crystal was then picked up in the cryoloop and flash-cooled in a stream of cold nitrogen gas at 100 K. If ice rings are detected in the diffraction pattern, the soaking time should be extended from 10 to 30 s and the remaining mother liquor should be removed more completely.

The experimental procedure for the water-based cryoprotectant was as follows. A protein crystal was introduced into 1.0–2.0 µl of the water-based cryoprotectant using a cryoloop and soaked for 5–20 s. The soaking time was optimized from crystal to crystal. If cracking or dissolution of the crystal was observed, quick-dip soaking for 1–5 s was tried (see §3.1).

2.3. Preparation and usage of heavy atoms in cryoprotectant

The compositions of the oil- and water-based cryoprotectants for heavy-atom derivatization were established through the derivatization experiments shown in Table 1. The oil-based

Table 2
Diffraction data.

Protein ID	No. of amino acids	Resolution limit (Å)/mosaicity (°)					Solvent content (%)
		Type I†	Type II‡	Type III§	Type IV¶	Type V††	
00367	177	2.1/0.92	2.1/0.82	1.9/0.61	1.9/0.39	1.8/0.44 (30%)	54.7
10017	153	No diffraction	nd‡‡	No diffraction	3.3/0.38	4.3/0.49 (30%)	62.5
00126	126	No diffraction, ice formation	nd	4.8/1.19, ice formation	2.1/0.41	1.9/0.40 (30%)	58.2
11731	457	4.0/1.08	nd	2.4/0.42	2.7/0.88	2.5/0.66 (20%)	44.8
00069	227	4.3/2.04	nd	2.9/0.81	Dissolved	2.5/0.53 (17%)	32.9–73.2§§

† Paratone-N with 10% glycerol (Kwong & Liu, 1999). ‡ Dry paraffin oil (Riboldi-Tunnicliffe & Hilgenfeld, 1999). § Oil-based cryoprotectant. ¶ Water-based cryoprotectant. †† Crystallization solution with 17–30% glycerol. Values in parentheses refer to the concentrations of glycerol used [(*w*/*v*)]. ‡‡ nd, not determined. §§ The range of solvent content for ID00069 was calculated from number of molecules (2–5) that could be present in the asymmetric unit (Matthews, 1968, 1976).

cryoprotectant mixture for heavy-atom derivatization consists of 57.0–59.5%(*w*/*w*) Paratone-N, 24.0–25.5%(*w*/*w*) paraffin oil, 9–10%(*w*/*w*) glycerol and 5–10%(*w*/*w*) of an aqueous solution of heavy-atom reagent at 10–50 m*M*. The water-based cryoprotectant mixture consists of 0.13%(*w*/*v*) hyaluronic acid, 45%(*w*/*v*) glycerol, 1 m*M* dithiothreitol (DTT) and 5–10 m*M* heavy-atom aqueous solution. Here, DTT may be removed if it reacts with heavy-atom reagent. Three water-based cryoprotectant solutions using K_2PtCl_4, $HgCl_2$ and $KAu(CN)_2$ were prepared for the derivatization experiments and stored at 277 K.

The derivatization experiments were performed using crystals from three different proteins. A crystal was soaked with 50–100 mg (for the oil-based cryoprotectant) or 2–4 µl (for the water-based cryoprotectant) of the heavy atom in cryoprotectant for 120–300 s. The soaking time was changed according to the size of the crystal (Table 1) and the level of crystal damage such as cracking and dissolution. After completing the data collection, anomalous Patterson maps were calculated using the program *FFT* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). SAD/SIR phasing and initial model constructions were performed using the programs *SOLVE*/*RESOLVE* (Terwilliger & Berendzen, 1999).

3. Results

3.1. Novel versatile cryoprotectants

With the novel oil-based cryoprotectant, crystal localization in the cryoloop can be clearly visualized compared with a conventional versatile oil-based cryoprotectant mixture (Paratone-N with 10% glycerol), which helps in centring the crystal in the X-ray beam (Fig. 1). In order to evaluate the versatility of our oil- and water-based cryoprotectants, we examined the cryoprotective abilities of the novel cryoprotectants on five proteins from *T. thermophilus* HB8 and *P. horikoshii* OT3 (Table 2). The cryoprotectants used were Paratone-N containing 10% glycerol (type I; Kwong & Liu, 1999), dry paraffin oil (type II; Riboldi-Tunnicliffe & Hilgenfeld, 1999), the novel oil-based cryoprotectant (type III), the novel water-based cryoprotectant (type IV) and the crystallization solution diluted with glycerol at 17–30%(*w*/*v*) concentration (type V). The glycerol concentrations of type V were optimized so as to give the best values of resolution and mosaicity. Thus, the result of the type V cryoprotectant was used as a reference for the evaluation of the other cryoprotectants. Here, type V cryoprotectants were made by dilution of the mother liquor with glycerol rather than the widely accepted method in which the water in the mother liquor is replaced by glycerol in order to keep the concentration of the mother-liquor components constant. However, we adopted this dilution method with glycerol to orient the large-scale crystal screening in structural genomics.

Three crystals from protein ID00367 were treated with type V cryoprotectant. They diffracted to 1.8 Å resolution and had a mosaicity of 0.44°. Crystals treated with type III and IV protectants diffracted to comparable resolutions. The mosaicities from type I and II protectants were a little higher than those from the type III, IV and V protectants. This result shows that type III and type IV protectants are suitable for the cryoprotection of this protein crystal.

In the case of ID10017, crystals treated with type I and III protectants did not diffract X-rays, indicating that Paratone-N caused physical damage to the crystals. Interestingly, the resolution and mosaicity from the type IV protectant were better than those from the type V protectant. In the protection of ID00126 crystals, the resolution and mosaicity from only type IV protectant were the same as those from type V protectant. Type I and III protectants led to the formation of an ice ring and the crystals suffered serious damage, leading to an unacceptable increase in mosaic spread from 0.40 to 1.19°. The crystals of ID10017 and ID00126 were damaged by oil-based cryoprotectants. In such cases, water-based type IV protectant gave better results. To minimize damage to the crystal during soaking with type IV protectants, a quick-dip soaking in which the crystals were exposed to the cryoprotectant for a brief period was effective in the cases of protein ID11055 and ID10950 (data not shown). This quick-dip soaking method with type IV protectant is often useful when the crystal is not stable in the cryoprotectant.

In the case of ID11731, crystals treated with the type III protectant exhibited a better resolution limit than those from the other protectants and the mosaic spread was better than

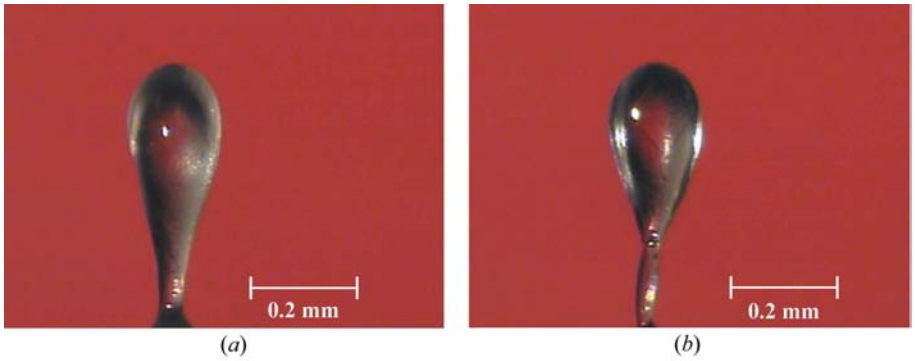

Figure 1
Photograph of a mounted flash-cooled crystal. The crystal was mounted using (*a*) Paratone-N with 10% glycerol and (*b*) the novel oil-based cryoprotectant. The crystal localization in (*b*) can be clearly visualized compared with (*a*).

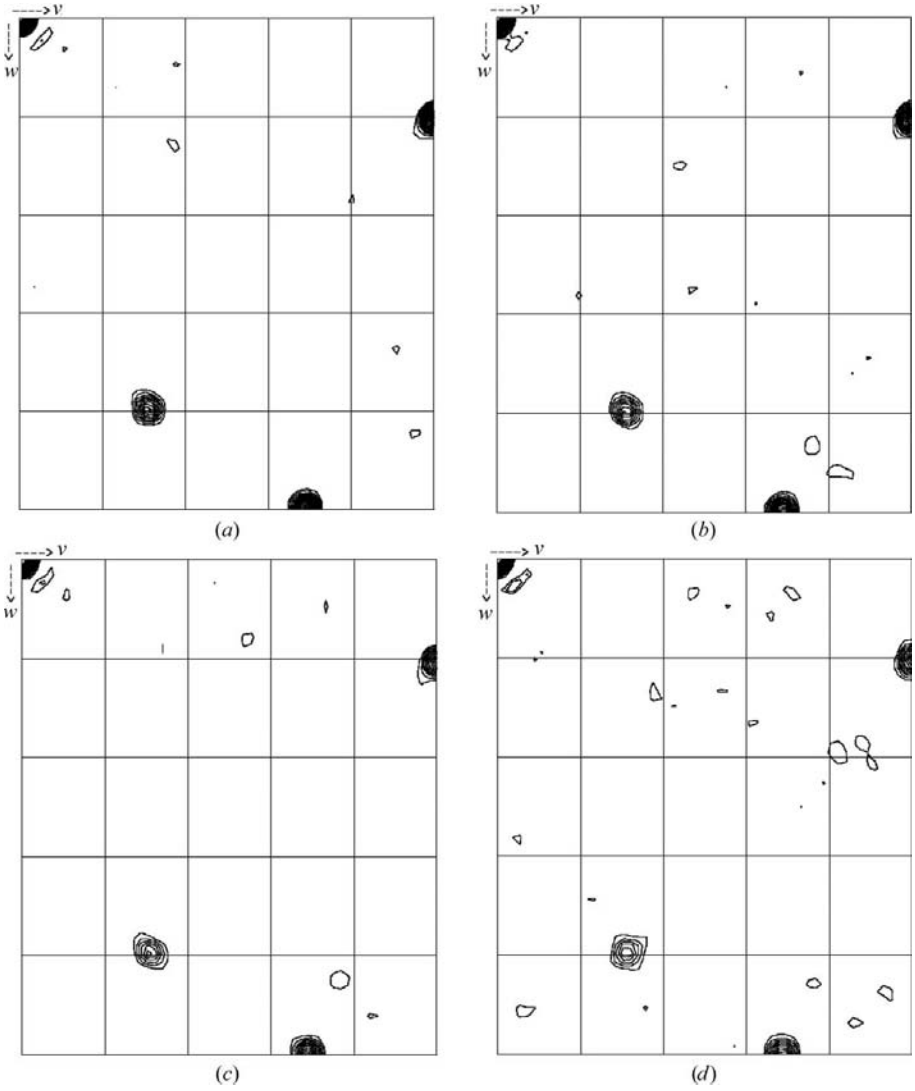

Figure 2
Anomalous Patterson maps of K_2PtCl_4 derivative of ID00367 contoured by 2σ (starting at the $+2\sigma$ level). The Harker sections ($u = 0$) at 2.5 Å resolution are shown. The crystals were derivatized using the heavy atom in oil-based cryoprotectant after 0 d (*a*) and 35 d (*b*) and the heavy atom in water-based cryoprotectant after 0 d (*c*) and 180 d (*d*) after preparation. The crystals belong to space group *I*222.

that from the type V protectant. In the case of ID00069, the resolution limit from the type III protectant was also better than that of the type I protectant. For the type I and III protectants, an increase in the mosaicity of more than 0.28° was observed compared with type V. Type IV cryoprotectant caused the dissolution of the crystal. From the results on ID11731 and ID00069, it may be concluded that the lower glycerol concentration in the type III protectant, ensuring a low osmotic gradient between the interior of crystal and the surrounding solution, made it suitable for the cryoprotection of these crystals.

3.2. Application to heavy-atom screening

Two cryoprotectant solutions were tested for effective screening of heavy-atom derivatization of protein crystals. In order to evaluate the heavy atom in cryoprotectants, we tested the derivatization of crystals on the three proteins from *T. thermophilus* HB8 and *P. horikoshii* OT3. The derivatization data for one crystal from each condition are shown in Table 1.

The derivatization of an ID00367 crystal was initially tried using the oil-based cryoprotectant diluted with 10 m*M* K_2PtCl_4 aqueous solution [63%(*w*/*w*) Paratone-N, 27%(*w*/*w*) paraffin oil, 5%(*w*/*w*) glycerol and 5%(*w*/*w*) 10 m*M* K_2PtCl_4] by soaking the crystal for 240 s. However, ice-ring formation was observed. Increasing the glycerol concentration of the cryoprotectant from 5 to 10% alleviated the problem of ice-ring formation and the ID00367 crystal was successfully derivatized. Prominent platinum peaks appeared on the Harker section of an anomalous Patterson map (Fig. 2*a*). Attempts at phasing and initial model building using the programs *SOLVE*/*RESOLVE* (Terwilliger & Berendzen, 1999) automatically yielded an initial model for over 70% of the structure. The final composition suitable for the platinum derivatization of this crystal was 59.5%(*w*/*w*) Paratone-N, 25.5%(*w*/*w*) paraffin oil, 10%(*w*/*w*) glycerol and 5%(*w*/*w*) of a 10 m*M* aqueous solution of K_2PtCl_4. In order to

evaluate the lifetime of the heavy-atom reagents in the oil-based cryoprotectant, we tried long-term storage of the solutions for over one month. Attempts to derivatize the crystal after 20 d failed with K_2PtCl_4 in the oil-based cryoprotectant. However, the derivatization was successful even after 35 d if the reagent concentration was increased to contain 10% of 50 m*M* heavy-atom reagent (Fig. 2*b*). An initial model for over 70% of the structure was also automatically constructed as in the case of the freshly prepared reagent.

The derivatization of crystals from three proteins were also attempted using water-based cryoprotectant in the presence of various heavy-atom reagents: K_2PtCl_4, $HgCl_2$, $KAu(CN)_2$ and $K_2Pt(CN)_4$ (Table 1). Here, the derivatization was tried using one crystal for each condition. In the derivatization of an ID00367 crystal using K_2PtCl_4 at a concentration of 10 m*M* and with a soaking time of 300 s, the platinum sites were confirmed in an anomalous Patterson map on the Harker section (Fig. 1*c*). ID00126 crystals were also successfully derivatized in $HgCl_2$ and $KAu(CN)_2$ at 10 m*M* heavy-atom concentration. Phasing and initial model building resulted in models for over 70% of the structure. In order to evaluate the lifetime of the heavy-atom reagents in water-based cryoprotectant, we again tried a long-term storage test. An ID00367 crystal was derivatized with K_2PtCl_4 cryoprotectant six months after the preparation of the reagent (Fig. 2*d*). The derivatizations trials of ID00126 and ID10754 crystals succeeded using $HgCl_2$, $KAu(CN)_2$ and $K_2Pt(CN)_4$ in water-based cryoprotectants that had been stored for one month. Initial models of over 70% of the structures were also automatically constructed. From these results, it may be concluded that hyaluronic acid extends the lifetime of the heavy-atom reagents. However, the reason for the extended lifetime remains uncertain. In an attempt to probe the optimal concentration range of heavy-atom reagents in the water-based cryoprotectant with ID00367 crystals, it was observed that the derivatizations succeeded with heavy-atom concentrations of 5 and 10 m*M*, but not at 1 m*M*. Therefore, the optimal heavy-atom concentration range of water-based cryoprotectant for the examined crystals might be from 5 to 10 m*M*.

4. Discussion

With conventional cryoprotectants such as glycerol in the crystallization solution, flash-cooling requires quick and trouble-free transfer of the loop-mounted crystal to the goniometer head of the diffractometer to avoid dehydration of the crystal. The present versatile cryoprotectants (oil- and water-based types) solve part of this problem, because the versatile cryoprotectants serve as a shield from dehydration for crystals during handling. The moderate viscosity of the novel oil-based cryoprotectant containing paraffin oil is lower than that of 100% Paratone-N, which improves the visibility of the mounted crystal in the cryoloop and is advantageous for removing the excess cryoprotectant solution from the loop. The smaller sample size was reported to be helpful for successful flash-cooling of protein crystals in terms of cooling rate (Chinte *et al.*, 2005), suggesting another advantage of reducing the protectant volume surrounding the crystal. The dry paraffin oil (Riboldi-Tunnicliffe & Hilgenfeld, 1999) may be also effective as an oil-based versatile cryoprotectant, although it cannot be applied to the heavy-atom derivatization of protein crystals. The novel water-based cryoprotectant might be useful for the cryoprotection of a variety of different protein crystals. It should be noted that the water-based cryoprotectant comprises only hyaluronic acid solution and glycerol. Further investigation of other hydrophilic polymers as potential cryoprotectant agents would be worthwhile.

Although the conventional type V cryoprotectant composed of glycerol and crystallization solution gives good results in most cases, the resolution limit and mosaicity from type IV (water-based) for ID10017 and type III (oil-based) for ID11731 were better than those from the type V protectant. The present results confirm that complementary use of both the oil- and water-based cryoprotectants allows sufficient cryoprotection by flash-cooling, indicating that the novel versatile cryoprotectants may serve to improve the efficiency of large-scale crystal screening in structural genomics initiatives. Interestingly, satisfactory results were obtained with the water-based cryoprotectant for crystals with high solvent contents of over 54.7%, while oil-based cryoprotectant gave better results for crystals with the lower solvent contents (Table 2). ID00367 crystals with a medium solvent content of 54.7% were successfully protected using both types of cryoprotectants. Type III oil-based cryoprotectants do not adequately penetrate crystals of high solvent content, in contrast to water-based and conventional cryoprotectants which penetrate the lattice. For high-solvent crystals, cryoprotection cannot be achieved without using penetrating cryoprotectant agents. From these results, it appears that the solvent content of crystals may be relevant to the choice of cryoprotectants, indicating that the properties of protein crystals such as contraction of the unit cell and solvent channels on cooling should be considered (Weik *et al.*, 2001; Juers & Matthews, 2004).

The results of the derivatization experiments show that cryoprotectant containing heavy atoms is useful for rapid and efficient cryoprotection and derivatization of crystals of three different proteins. The heavy atom in cryoprotectant solutions could be stored with sufficient derivatizing ability for over one month at 277 K. The disadvantages of this derivatization method may be unavailability of back-soaking and bound glycerol. Although back-soaking should give less background and reduced radiation damage (Garman & Murray, 2003), all data sets were collected from derivatized crystals without back-soaking. Having glycerol in the cryoprotectant sometimes results in its binding to specific sites on the protein molecular surface in crystals (Gregoriou *et al.*, 1998), which could act to inhibit the heavy-atom binding if it competes with glycerol. This problem might be solved if the glycerol in the versatile cryoprotectants is replaced by other cryoprotectant agents (*e.g.* MPD as in Gregoriou *et al.*, 1998). Despite these potential problems, this derivatization method may be useful for large-scale screening in structural genomics efforts, since it

can skip laborious and time-consuming processes such as the necessity for fresh preparation of reagents and back-soaking. In addition, a list of useful heavy-atom reagents is available in the program *HATODAS*, which is a web-based heavy-atom database system supporting the derivatization of protein crystals (Sugahara *et al.*, 2005; http://hatodas.harima.riken.jp/). The combined use of *HATODAS* and heavy atoms in cryoprotectant solutions may contribute to more efficient structural genomics pipelines.

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