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Macromolecular crystal annealing: evaluation of techniques and variables

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Additional examples of successful application of macromolecular crystal annealing are presented. A qualitative evaluation of variables related to the annealing process was conducted using a variety of macromolecular crystals to determine in which cases parameters may be varied and in which cases the original macromolecular crystal annealing protocol is preferred. A hypothesis is presented relating the solvent content of the crystal to the specific protocol necessary for the successful application of annealing.

1. Macromolecular crystal annealing

The rewarming of a flash-cooled crystal to reduce mosaicity and potentially increase resolution was introduced at the 1997 American Crystallographic Association meeting (Harp *et al.*, 1997) and was discussed in detail in a recent publication (Harp *et al.*, 1998). Briefly, the technique of macromolecular crystal annealing (MCA) is to remove a flash-cooled crystal from the cold gas stream of the cryostat and quickly place it in 300 µl of its cryoprotectant solution, and after 3 min reflash-cool it in the cold gas stream. In our first report on this subject (Harp *et al.*, 1998), we presented quantitative data demonstrating improvements in diffraction quality after macromolecular crystal annealing of nucleosome core particle (NCP) and chicken histone octamer (CHO) crystals as well as a lysozyme crystal that underwent multiple cycles of MCA without damage and without affecting the molecular structure. The present study is a qualitative assessment of the mechanics of annealing covering a broad range of macromolecules. The data presented here concern the success of annealing defined as the lack of deterioration of observed diffraction patterns after subjecting a crystal to an annealing treatment. This has allowed a rapid evaluation of the parameters involved in the application of annealing such as incubation time, crystal size, and dehydration effects. It has also allowed us to formulate a hypothesis concerning which crystals are best annealed by the protocol reported in our original paper and when parameters such as incubation time may be changed.

Not all crystals will exhibit increased mosaicity associated with flash-cooling and will not be improved by annealing. However, the MCA protocol provides an option that can be excercised where initial diffraction quality of a crystal is unsatisfactory. Since the original report on MCA a number of new crystal systems have been reported to be successfully annealed. Table 1 summarizes six crystal systems, in addition to the ones investigated in this study, which have been reported to exhibit improved diffraction after MCA treatment.

Table 1
Crystal systems that have been successfully annealed in addition to those used in this study.

	Space group	Unit-cell dimensions (Å, °)	Precipitant	Cryoprotectant
Fumarylacetoacetate hydrolase (unpublished data)	$P2_1$	$a = 64.0$ $b = 11.0$ $c = 67.0$ $\beta = 102.0$	PEG 8000	PEG 400
Alcohol dehydrogenase (T. Hurley, personal communication)	$P2_1$	$a = 55.7$ $b = 100.2$ $c = 69.0$ $\beta = 104.9$	PEG 6000	PEG 200
Aldehyde dehydrogenase (T. Hurley, personal communication)	$P2_1$	$a = 102.0$ $b = 177.0$ $c = 101.0$ $\beta = 94.6$	$MnCl_2$, MPD	Ethylene glycol
Crotonase (T. Hurley, personal communication)	$P3_1$	$a = 76.5$ $b = 76.5$ $c = 214.6$	PEG, NaCl	MPD
Pyruvate dehydrogenase kinase (N. Steussy, personal communication)	$P2$	$a = 72.0$ $b = 109.0$ $c = 73.0$ $\beta = 102.0$	—	Paratone N
Purine operon repressor (S. Sinha, personal communication)	$P1$	$a = 65.4$ $b = 72.5$ $c = 83.4$ $\alpha = 84.7$ $\beta = 84.6$ $\gamma = 67.5$		

2. Annealing on the loop

A recent fast communication (Yeh & Hol, 1998), published prior to our manuscript of the MCA method, described a variation of crystal annealing called flash-annealing by the authors. This method differs from ours by leaving the crystal in the cryoloop. The crystal is annealed *in situ* by first diverting the cold gas stream for 1.5–2.0 s, then reflash-cooling and allowing the crystal to recool for 6 s before repeating the process. The published procedure calls for three rounds of rewarming and flash-cooling. The communication mentions the succesful implementation of the technique with crystals of one protein, glycerol kinase.

In our laboratory, MCA has been successful in all cases where the crystal is stable in the cryoprotectant solution. Yeh and Hol report that our method did not work for glycerol kinase crystals. However, their use of 50 µl instead of the prescribed 300 µl of cryoprotectant solution suggests that the MCA protocol was not strictly followed. In our original report, we referenced Sauer & Ceska (1997) who, similar to the results of Yeh and Hol, indicated that some flash-cooled protein crystals could be warmed and reflash-cooled on the loop without loss of diffraction. In our initial investigations of the phenonmenon of annealing, we investigated what we have termed annealing on the loop. NCP and CHO crystals were both tried with this technique and, although we had occasional success with NCP crystals, we found the procedure inconsistent and difficult to control. The MCA protocol was developed to provide consistent results. However, we felt it would be useful to re-examine annealing on the loop in light of the results of Yeh and Hol. We have been careful to distinguish between annealing on the loop and the flash-annealing protocol presented by Yeh & Hol (1998). The protocols differ in two important respects: incubation time or the time allowed for warming of a flash-cooled crystal before reflash-cooling, and iterations of the warming and reflash-cooling cycles. Flash-annealing is taken here as requiring a warming time of 1.5 to 2.0 s and three rounds of warming and reflash-cooling as promulgated by Yeh and Hol. Annealing on the loop involves a variable length of time for warming and does not require multiple rounds of warming and reflash-cooling. Table 2 lists the crystals used in the comparative studies.

3. Data collection

Concanavalin A, chicken-egg lysozyme and proteinase K were acquired from Sigma Chemical, St Louis, MO, USA. Sperm whale myoglobin was provided by Allen Edmundson, Oklahoma Medical Research Foundation. Chicken histone octamer and nucleosome core particles were produced from recombinant components. Commercially acquired samples and sperm whale myoglobin were dialysed directly into crystallization buffers. The purification of chicken histone octamer and nucleosome core particle are detailed in Harp *et al.* (1996).

Crystals were grown using published protocols as obtained from the Biological Macromolecular Crystallization Database (Gilliland *et al.*, 1994). The crystals were mounted on rayon loops using the Hampton CrystalCap system (Hampton Research, Riverside, CA, USA) and were flash-cooled directly in the cold nitrogen gas stream. The cryostat consisted of a transfer tube (Molecular Structure Corp, The Woodlands, TX, USA) delivering a 90 K cold nitrogen gas stream coaxial with the spindle. Diffraction images were collected on an 18 cm Mar image-plate area detector (X-ray Research Gmbh) and a Rigaku RU-200 rotating anode X-ray generator with a copper anode and double focussing mirrors (Charles Supper Co., Natick, MA, USA).

Crystals were mounted on rayon loops on magnetic goniometer caps (Hampton Research, Riverside, CA, USA) using loops individually chosen to fit the dimensions of the crystal. The goniometer cap with the loop was securely attached to the goniometer while diverting the cold nitrogen gas stream and the mounted crystal was then flash-cooled by quickly removing the card used to divert the nitrogen gas stream. In those cases when excess liquid was wicked from the crystal prior to flash-cooling, the crystal was lifted from the cryoprotectant solution using the rayon loop on the magnetic

Table 2
Crystal systems used for evaluation of variables associated with the annealing.

	Solvent fraction	Space group	Unit-cell dimensions (Å, °)	Cryoprotectant
Concanavalin A	0.49	$I222$	a = 89.6 b = 86.5 c = 62.1	MPD/glycerol/ Paratone N
Proteinase K	0.46	$P4_32_12$	$a = b$ = 68.3 c = 108.4	Glycerol
Nucleosome core particle	0.51	$P2_12_12_1$	a = 105 b = 110 c = 180	MPD
Chicken histone octamer	0.65	$P3_221$	$a = b$ = 115 c = 100	Glycerol
Lysozyme (± *N*-acetyl glucosamine)	0.37	$P4_32_12$	$a = b$ = 78.4 c = 37	Glycerol
Sperm whale myoglobin	0.34	$P2_1$	a = 64.7 b = 30.8 c = 35.1 β = 106.3	Glycerol

goniometer cap and an absorbent paper dental point was used as a wick to draw free liquid from the loop.

Diffraction images were collected before and after annealing treatments at different orientations corresponding to rotation angles of 0, 30, 45, 60 and 90° to establish diffraction quality. Each crystal was then annealed on the loop or subjected to MCA, and diffraction images collected to assess the response of the crystal to the treatment. Success of an annealing treatment was judged qualitatively by visual examination of diffraction images taken before and after the treatment. Success was recorded when the annealing treatment produced no discernable deterioration in diffraction quality.

Annealing was carried out by MCA carefully following the protocol as originally described (Harp *et al.,* 1997). Flash-annealing was carried out following the protocol exactly as described by Yeh & Hol (1998). For annealing on the loop, warming times were adjusted by visually monitoring the crystal using the video camera provided with the Mar image-plate area detector. Flash-cooled crystals quickly become covered in ice when the cold nitrogen gas stream is diverted and then clear as the crystal warms. Warming time for a crystal was recorded as the time elapsed between diversion of the cold nitrogen gas stream and visual clearing of the crystal on the loop. The corrected warming time is proportional to the size of the crystal.

4. Observation of specific crystals

4.1. Sperm whale myoglobin

Sperm whale myoglobin crystals (Tilton *et al.,* 1984) were initially flash-cooled using 30% glycerol in the reservoir solution. The crystals grew as plates with a minimum dimension of 0.1–0.15 mm. Flash-annealing was not successful with the myoglobin crystals since the crystals did not clear within the 2.0 s warming time. Crystals were successfully annealed on the loop. MCA was also successful and crystals annealed on the loop could subsequently be given MCA treatment with no apparent degradation in diffraction quality. The very low solvent content of the myoglobin crystals, 34% (Matthews, 1968), may account for this ease of handling and flash-cooling. Myoglobin crystals do not appear to suffer from increased mosaicity after initial flash-cooling.

4.2. Chicken egg lysozyme

Lysozyme and lysozyme bound with *N*-acetyl glucosamine, NAG, crystals (Hadfield *et al.,* 1994) were flash-cooled using 30% glycerol in reservoir solution. As shown previously (Harp *et al.,* 1998) there is little or no increase in mosaicity associated with the initial flash-cooling of lysozyme crystals and lysozyme/NAG crystals appear to be quite similar in behavior. Flash-annealing was not successful since the crystals did not clear within the 2.0 s warming time. Annealing on the loop was generally successful with these crystals. It was found that annealing on the loop was more consistent if all surface solution was wicked from the crystal using an absorbent paper dental point. The solvent content of lysozyme crystals was calculated as 37%.

4.3. Nucleosome core particle

The nucleosome core particle crystals grow as hexagonal rods as previously described (Harp *et al.,* 1998). Crystals used for annealing on the loop were 0.1–0.2 mm in diameter. The cryoprotectant for the initial flash-cooling was 22.5% MPD, 30 m*M* $MnCl_2$, 30 m*M* KCl, 20 m*M* K-cacodylate pH 6.0. The solvent content of nucleosome crystals is approximately 51%. The crystals exhibit significant increase in mosaicity on initial flash-cooling. Application of flash-annealing to NCP crystals was not successful since clearing times were always longer than 2.0 s. Even with adjusted warming times, the nucleosome core particle crystals did not anneal on the loop successfully unless all surface solution was wicked away using a dental point after mounting the crystal on the loop. Annealing on the loop after wicking of the solution from the crystal was inconsistent because the nucleosome core particle crystals are sensitive to dehydration. For initial flash-cooling, minimization of the time between removing the crystals from the cryoprotectant solution and flash-cooling is critical. Also, in several instances, spurious strong diffraction spots were apparent in diffraction images after three iterations of annealing on the loop, as is shown in Fig. 1. The reflections were typical of diffraction from inorganic crystallites. The crystallites apparently formed on the surface of the NCP crystals due to drying during the warming periods. The reflections were not

apparent in diffraction images prior to the third annealing on the loop cycle.

4.4. Chicken histone octamer

Chicken histone octamer crystals grow as hexagonal bipyramids (Burlingame *et al.,* 1984) and are routinely 0.5 mm or greater in diameter. Cryoprotection is achieved by dialysis into 15% glycerol in crystal storage buffer (71% saturated ammonium sulfate, 20 m*M* pyrophosphate, 5 m*M* EDTA, 10 m*M* β-mercaptoethanol). Histone octamer crystals can undergo MCA without problems but are consistently damaged by the annealing on the loop protocol. CHO crystals could not be flash-annealed as the ice did not clear within 2.0 s. During the annealing on the loop protocol crystals were observed to clear during the first warming period then become cloudy upon reflash-cooling. Diffraction after reflash-cooling was significantly degraded. In our original investigation, MCA reduced mosaicity of CHO diffraction by 20–30%. Even multiple rounds of MCA did not significantly degrade CHO diffraction quality while only a single treatment by annealing on the loop or of flash-annealing caused irreversible damage. Fig. 2 illustrates this with a 0.4 × 0.4 × 1.0 mm crystal. Initial diffraction (Fig. 2*a*) was adequate and after three rounds of MCA (Fig. 2*b*) diffraction is still adequate. Fig. 2(*c*) shows the loss of diffraction quality in the same crystal after annealing on the loop. The initial diffraction quality of the crystal could not be restored by subsequent MCA. To investigate the effect of size on the response of CHO crystals to annealing on the loop, another histone crystal was cut to produce a fragment with smallest dimension of about 0.1 mm. Annealing on the loop was not successful on the small crystal fragment.

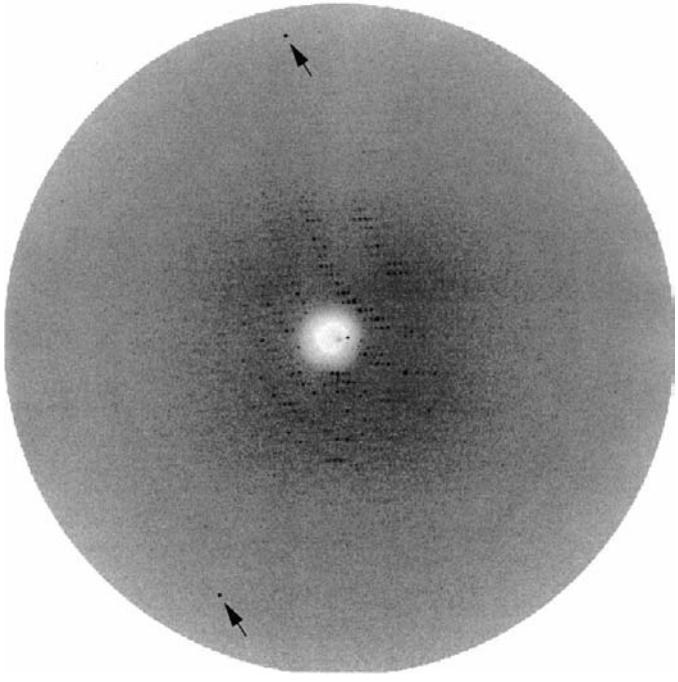

Figure 1
Diffraction image taken from a nucleosome core particle crystal after flash-annealing. Strong, spurious diffraction spots (arrows) appear after the third round of warming and flash-cooling.

4.5. Proteinase K

Crystals of proteinase K (Wolf *et al.,* 1991) were grown as small tetragonal bipyramids about 0.1 × 0.15 mm. The crystals were intially flash-cooled in 30% glycerol, 50 m*M* Tris–HCl, pH 7.5. The crystals were successfully annealed using the flash-annealing protocol as given in Yeh & Hol (1998) without modification as well as by annealing on the loop. MCA was also successful. Slight ice-rings were still apparent in the flash-annealed diffraction images.

4.6. Concanavalin A

Concanavalin A crystals were grown from 0.1 *M* $NaNO_3$, 50 m*M* Tris–acetate, pH 6.5 [Kalb (Gilboa) *et al.,* 1988] with smallest dimension about 0.2–0.3 mm. Initial flash-cooling used either 30% glycerol or 25% MPD in 1–2 min soaks. In either case, initial flash-cooling provided adequate diffraction but neither flash-annealing or annealing on the loop were successful. In the application of MCA, the crystals were not stable in the cryoprotectant and became opaque during the room temperature incubation. Thus, the concanavalin A crystals could not be annealed by MCA. Paratone N was also tried as cryoprotectant but again the crystals could not be flash-annealed or annealed on the loop. An adequate cryoprotectant solution remains to be found for crystals of this protein. Crystals of concanavalin A are known to undergo a phase transition near 165 K (Walker *et al.,* 1998) which may disorder the crystal lattice during the warming from 100 K to room temperature. This is the first case in which a crystal has not been successfully annealed by MCA in this laboratory.

5. General observations and discussion

It is apparent that for flash-annealing, the 2.0 s warming time specified by Yeh and Hol was inadequate for most crystals and usually resulted in severe icing. Warming times for annealing on the loop were established empirically by observation of the crystal using the video microscope provided with the Mar image-plate area detector. When the cold nitrogen gas stream is blocked, the crystal first becomes opaque and then undergoes clearing. The time to clearing was measured and used for subsequent cycles to investigate the effect of multiple cycles of annealing on the loop. Surprisingly, the time to clearing of the crystal often increased substantially in subsequent cycles of annealing on the loop, complicating control of the procedure. From our experiments, three cycles of annealing on the loop is not needed. When diffraction improvements were made, they occurred on the first cycle. Moreover, the thawing time had to be established empirically for each crystal examined.

Wicking of the solution from the surface of the crystal after mounting on the loop made the flash-annealing protocol more likely to succeed. However, wicking increases the difficulty of mounting the crystal and can cause deterioration of crystal quality by drying during and after mounting on the loop. This

was particularly true when working with nucleosome core particle crystals.

There are, however, other factors besides size. Our results with a variety of crystals (Table 3) suggest that the solvent content is correlated with the length of incubation time needed for annealing. That is, whether or not incubation in cryoprotectant in the MCA protocol is required. Some crystals may anneal quickly during the few seconds needed for clearing of ice in flash-annealing or annealing on the loop protocols. Myoglobin (34% solvent fraction) and lysozyme (37% solvent fraction) crystals are not significantly affected by flash-cooling and may be successfully annealed on the loop as well as by MCA. These crystals possessed the lowest solvent content of any in this study. The crystals of the nucleosome core particle (51% solvent fraction) showed a moderate dependency on warming time so that annealing on the loop is possible but not dependable. The MCA protocol is used for consistent results. The histone octamer crystals (65% solvent fraction) showed the greatest dependency on warming time for successful annealing. These crystals can only be successfully annealed by the 3 min of incubation in the MCA protocol which was originally developed using the CHO crystals. A 2.5 min incubation was not sufficient for consistent results. The lack of success for annealing on the loop in the case of the histone octamer is likely the result of high solvent content. The size of the CHO crystals was not a factor, since annealing on the loop was unsuccessful on small, 0.1 mm, crystals as well as larger, 0.5–1.0 mm, crystals. The CHO crystals have a typical mosaicity (0.2°) when data are collected at 277 K and are not inherently highly mosaic.

The success of the annealing on the loop protocol was made more consistent by wicking excess solution from the crystal after mounting on the loop. However, wicking increases the difficulty of crystal mounting and may result in unacceptable drying of the crystal. Excessive drying of crystals can result in increased mosaicity and lower resolution (Teng & Moffat, 1998). The issue of crystal drying was not addressed by Yeh

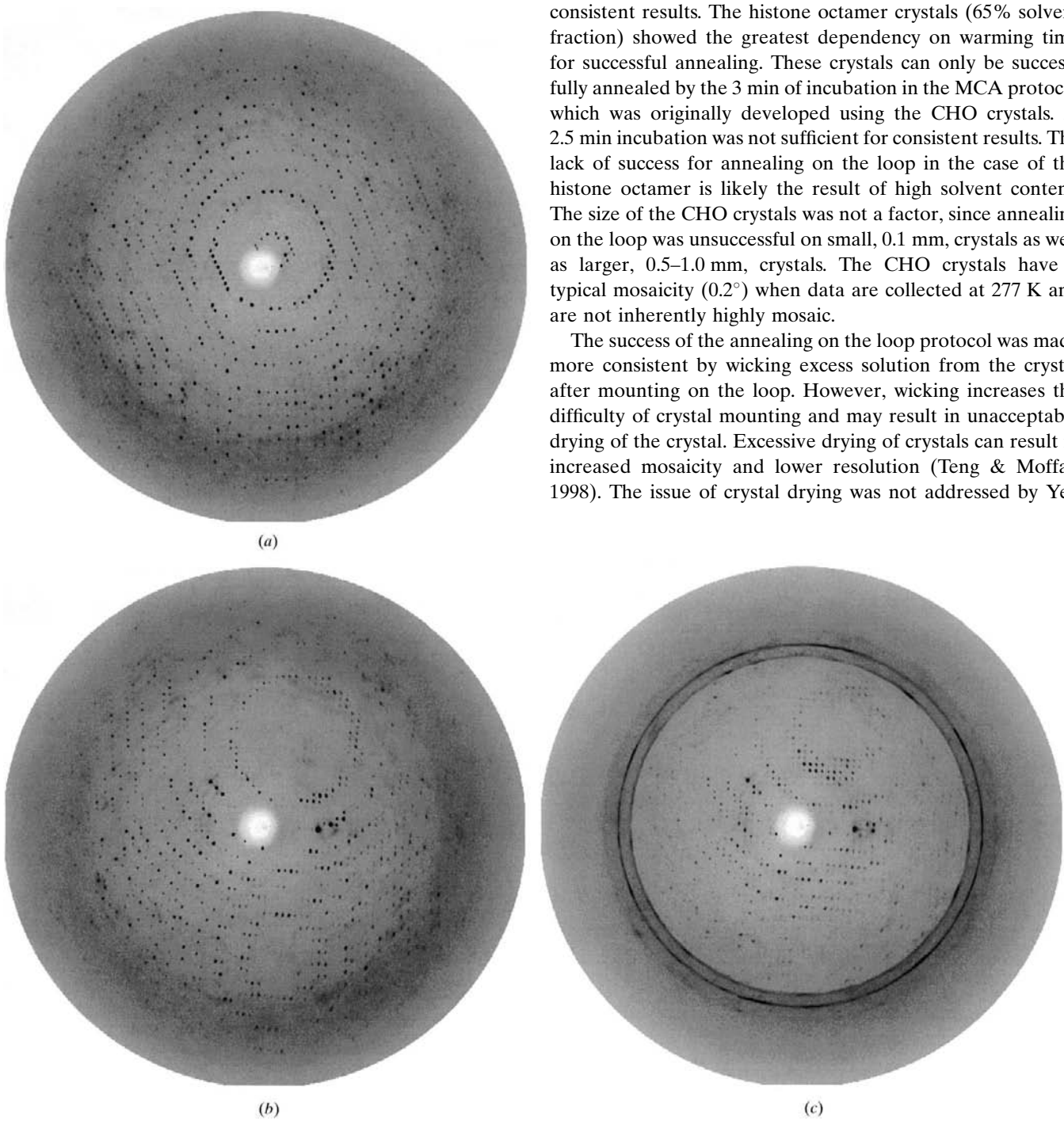

Figure 2
Diffraction images taken from a histone octamer crystal to demonstrate the effect of: (*a*) initial flash-cooling, (*b*) three rounds of macromolecular crystal annealing and (*c*) subsequent flash-annealing of the same crystal. Diffuse scatter evident in these diffraction images is typical for CHO and arises from disordered tail regions which account for about 25% of the protein mass.

and Hol although the statement was made that increasing the thawing time by only a few seconds was detrimental to both the mosaicity and diffraction limit. This result was interpreted by them as being related only to the warming of the crystal. Yeh and Hol suggest that increased room temperature incubation could be detrimental to diffraction quality. We propose that crystal drying may be the factor contributing to loss of diffraction quality when thawing time was increased for the glycerol kinase crystals. Also, in our observations, spurious strong diffraction spots appeared in some cases after multiple rounds of annealing on the loop using nucleosome core particle crystals. These spots appear typical of diffraction from inorganic crystallites. The spurious reflections were not visible in initial flash-cooled images nor after one round of annealing on the loop and must have developed during the multi-cycle annealing procedure.

MCA has been successful in this laboratory on all but one crystal, concanavalin A. These crystals can be initially flash-cooled to provide adequate diffraction, but no solution yet found confers stability on soaking crystals in cryoprotectant at ambient temperatures for more than a minute or two. The dissolution of these crystals is even more rapid after flash-cooling.

From our experience, successful application of annealing on the loop is dependent on several factors. Small crystals have the best chance for successful annealing on the loop. The warming time for each crystal will be variable depending on the mass (size) of the crystal. To ensure adequate warming, the crystal must be clear before it is reflash-cooled. The crystal must also be 'dry' prior to annealing on the loop, and the procedure should be performed once, not multiple times. The best variable for predicting success is the solvent content of the crystal; the higher the solvent content, the less likely annealing on the loop will prove fruitful.

The protocol for macromolecular crystal annealing, as originally reported (Harp *et al.,* 1998), was developed to provide reproducible, successful annealing with all crystals examined. In every case in which an adequate cryoprotectant is available, the original protocol has performed as intended. The parameters can, in some cases, be modified to shorten incubation times or lower volumes of annealing solutions. However, the original protocol is still to be recommended when approaching a crystal system for which the response to annealing is unknown.[1]

[1] For the information of the reader: Harp and Bunick paper, *Acta Cryst.* D**54**, 622–628, 1998; received 18 September 1997, accepted 9 December 1997 (research paper). Yeh and Hol paper, *Acta Cryst.* D**54**, 479–480, 1998; received 28 February 1998, accepted 27 March 1998 (fast communication).

Table 3
Summary of the results of annealing treatments on the crystal systems used in this study.

Annealing on the loop was compared with standard macromolecular crystal annealing and related to the solvent content of the crystal unit cell. Results of annealing on the loop for the nucleosome core particle crystals required removal of surface liquid prior to flash-cooling for success. Success for an annealing protocol was defined as not producing a discernable deterioration in diffraction quality.

		Successful annealing	
Molecule	Solvent fraction	On the loop	MCA
Sperm whale myoglobin	0.34	Yes	Yes
Lysozyme (± *N*-acetyl glucosamine)	0.37	Yes	Yes
Proteinase K	0.46	Yes	Yes
Concanavalin A	0.49	No	No
Nucleosome core particle	0.51	No/Yes	Yes
Chicken histone octamer	0.65	No	Yes

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