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Improving the X-ray resolution by reversible flash-cooling combined with concentration screening, as exemplified with PPase

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A significant improvement in the X-ray resolution of crystals of *Escherichia coli* inorganic pyrophosphatase at cryo-temperature was obtained as a result of studying the relationship between the crystal order and cryosolution component concentrations. To perform the experiments, the ability to reverse the flash-cooling process and to return a crystal to ambient temperature was used. In each cycle, the crystal was transferred from a cold nitrogen-gas stream to a cryosolution with modified concentrations of the components. The crystal was then flash-cooled again and the diffraction quality checked. Such a technique allows the screening of a wide concentration range rather quickly without using a large number of crystals and allows the determination of optimal cryosolution component concentrations. The resolution limit for crystals of pyrophosphatase increased by almost 0.7 Å, from 1.8 to 1.15 Å.

1. Introduction

The reliability and accuracy of molecular structures obtained by X-ray crystal structure analysis depends mainly on the resolution of the data. Specific features of the diffraction patterns from protein crystals are weak scattering, a sharp fall-off of reflection intensity with the diffraction angle and high background scattering. For the majority of reflections, the net diffraction intensity is much less than the background itself and their ratio becomes progressively lower with increasing resolution. As a result, for macromolecular crystals it is not the physical limit of the diffraction method itself that determines the resolution ($d = \lambda/2$, where λ is the wavelength of the radiation used). It is just not feasible to obtain reliable count statistics within a reasonable time.

There are two ways to extend the resolution of X-ray diffraction patterns. The first involves further development of the instrumentation and the techniques of data collection, which would allow one to increase the radiation intensity and decrease the background scattering by using synchrotron-radiation sources of ever-increasing power and creating more and more perfect detectors (Helliwell, 1997). The main limitation is associated with radiation damage to the crystal, leading to its decay even at cryo-temperatures (Gonzalez & Nave, 1994).

The alternative method is to improve the diffraction quality of protein crystals themselves. Although protein crystals often have low mosaicity (Helliwell, 1988; Colapietro *et al.*, 1992; Fourme *et al.*, 1995), the decrease in their intensity with the diffraction angle is much more pronounced than for inorganic crystals. The sharp decrease in intensity (large *B*-factor value

characterizing thermal and static disorder in a crystal) in the case of macromolecules cannot be accounted for only by the presence of such crystal defects as twins, inclusions, vacancies, dislocations and amorphous sediment (Dobrianov *et al.*, 1998). The crystal disorder is caused both by the high degree of freedom of protein molecules and by their conformational flexibility (Fraunfelder, 1985). The crystals contain large amounts of water, salts and other small molecules present in the mother liquor. Even small variations in solvent concentration, humidity and pH can cause significant changes in molecular packing and crystal order (Frey, 1994). That is why any deviations from ideal equilibrium conditions during crystal growth may act as a disordering factor (Dobrianov *et al.*, 1998).

Until now, the main efforts towards improving order in protein crystals have been focused on the modification of known methods and development of new methods of crystal growth (Weber, 1997; Riès-Kautt & Ducruix, 1997; DeLucas *et al.*, 1989). The difficulty in determining optimal conditions for the crystallization of proteins is associated with a large number of growth-affecting parameters, some of them being virtually uncontrollable. As is evident from several papers published during recent years, one can however improve the diffraction quality of crystals grown already. Higher order in protein crystals has been achieved either by crystal dehydration or post-crystallization soaking in solutions containing stabilizing agents such as glycerol and polyols (Sousa, 1995). By monitoring the changes in the unit cell of HIV-1 reverse transcriptase crystals during controlled dehydration, Esnouf *et al.* (1998) managed to successfully determine conditions which improved the resolution from 3.7 to 2.2 Å. Earlier, an improvement in diffraction as a result of crystal partial dehydration was observed in crystals of a complex between cytochrome *c* and cytochrome *c* peroxidase (Pelletier & Kraut, 1992). It can be suggested that the change in the diffraction limit from 3.6 to 2.8 Å obtained using multiple flash-annealing of glycerol kinase crystals (Yeh & Hol, 1998) can be attributed to changes that occurred during uncontrolled dehydration. Rould *et al.* (1991) showed that soaking co-crystals of aminoacyl-tRNA synthetase–tRNA in 20% glycerol followed by cooling to 265 K increased their diffraction limit and the order in disordered regions of these crystals. Significant improvement in the resolution (from 3 Å to better than 2.0 Å) and X-ray diffraction quality of MTCP-1 protein crystals was observed upon post-crystallization soaking in a solution with an increased concentration of ammonium sulfate (Fu *et al.*, 1999).

Here, we describe a new method that was used to extend the diffraction limit of the *E. coli* inorganic pyrophosphatase (PPase). At ambient temperature, PPase crystals usually diffract to 2.0–1.8 Å resolution (Kankare *et al.*, 1996; Harutyunyan *et al.*, 1997; Avaeva *et al.*, 1998). Using flash-cooling with mother liquor containing 27–30%(*w*/*v*) glycerol providing cryoprotection led to a minor improvement in resolution, to 1.8–1.7 Å. However, during crystal screening we observed the effect of salt concentration in a buffered solution upon the diffraction limit of the PPase crystals.

To investigate this effect, we have used the ability to reverse the flash-cooling process and to return a crystal to ambient temperature (Sauer & Ceska, 1997; Harp *et al.*, 1998). As opposed to the method of macromolecular crystal annealing (Harp *et al.*, 1998), during warming the crystal was placed in a cryosolution with sequentially modified concentration of the components. Application of this method allowed us to determine the optimal salt concentration in a cryosolution that would provide PPase crystals which diffracted to a resolution of higher than 1.2 Å. An alternative method involved crystal soaking in artificial mother liquor with a gradually increasing salt concentration up to that close to saturation, followed by flash-cooling.

A variety of macromolecular crystals allowed multiple transitions from ambient to cryogenic temperature (Harp *et al.*, 1999) and could tolerate sequential soaking in changing concentrations of cryosolution (Rodgers, 1997; Garman, 1999). These observations led to the suggestion that repetitive flash-cooling combined with concentration screening might be generally applicable to study the relationship between the diffraction quality of protein crystals and the chemical properties of the solvent within the crystals.

2. Materials and methods

2.1. Crystallization and flash-cooling

Soluble inorganic pyrophosphatase catalyses the hydrolysis of inorganic pyrophosphate and provides a thermodynamic driving force for many biosynthetic reactions (Chen *et al.*, 1990). The relative simplicity of the substrate makes PPase a good system in which to study the mechanism of enzyme-catalysed phosphoryl transfer (Maegley *et al.*, 1996). *E. coli* PPase is a homohexamer containing 175 amino-acid residues per monomer and arranged as a dimer of two trimers. The crystals of *E. coli* PPase belong to the space group *R*32 and have one monomer in the asymmetric unit. The solvent content is approximately 40%.

The complex of PPase with Ca^{2+} was crystallized by equilibrating a hanging drop of equal volumes of protein and reservoir solution against a reservoir solution consisting of 0.2 *M* sodium acetate buffer pH 5.0, 1.4–2.0 *M* NaCl (Merck), 180 m*M* $CaCl_2$. Rhombohedral crystals grew in 2–3 weeks to dimensions of 0.4 × 0.4 × 0.3 mm.

The PPase crystals exhibit rather isotropic diffraction patterns. At room temperature, the mosaicity of PPase crystals is less then 0.3° and usually tends to be higher for flash-cooled crystals.

For cryomeasurements, the crystals were placed into mother liquor containing 27–30%(*w*/*v*) glycerol as cryoprotectant for 5–10 s, followed by immediate flash-cooling. The crystal was then removed from the cold nitrogen-gas stream and quickly transferred to the cryosolution drop. The crystal was kept in the cryosolution for 20–25 s before the next flash-cooling.

PPase crystals are 'typical' protein crystals, which are easily destroyed upon careless handling. Sudden changes in the concentrations in the cryosolution damages them. Therefore,

Table 1
Data-collection statistics.

The data sets tabulated here were obtained after improving the diffraction quality of the crystals by achieving the optimal cryosolution concentration by either flash-cooling cycles [PPase + Ca^{2+} (I) and PPase + Ca^{2+} + pyrophosphate] or soaking up to limiting NaCl concentrations [PPase + Ca^{2+} (II) and PPase + Mg^{2+}]. Values in parentheses are for the highest resolution shell.

	PPase + Ca^{2+} (I)	PPase + Ca^{2+} (II)	PPase + Ca^{2+} + pyrophosphate	PPase + Mg^{2+}
Space group	*R*32	*R*32	*R*32	*R*32
Unit-cell parameters				
a, *b* (Å)	109.52	109.34	109.37	109.17
c (Å)	75.08	74.76	75.59	75.11
Resolution (Å)	20.0–1.05	20.0–1.20	20.0–1.20	20.0–1.15
Measured reflections	662283	690683	640235	676576
Unique reflections	79833	53148	51915	60427
Completeness (%)	99.6 (99.3)	99.3 (96.7)	96.1 (91.7)	99.5 (99.8)
$\langle I/\sigma(I)\rangle$	15.9 (2.6)	14.0 (2.6)	17.1 (3.6)	14.0(2.9)
$R_{\rm merge}$ (%)	3.4 (44.2)	4.2 (52.3)	3.6 (47.0)	3.5 (53.3)

the NaCl concentration step sizes in cryosolution were not more than 0.1 *M*.

2.2. Data collection and processing

X-ray measurements were carried out at beamline X11 of the EMBL Outstation at DESY, Hamburg, at a wavelength of 0.906 Å with an X-ray Research (Hamburg) 345 mm image-plate area detector. Crystals were frozen at 100 K with an Oxford Cryosystems Cryostream using the loop-mounting method. The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Crystal mosaicity was evaluated by examining the intensity distribution of reflections with respect to oscillation angle using *DENZO* (Otwinowski & Minor, 1997). As an estimate of the crystal order, Wilson's *B* factor (Wilson, 1949) was used as calculated by *TRUNCATE* (Collaborative Computational Project, Number 4, 1994).

To check the diffraction quality of PPase crystals after every cycle of flash-cooling, 5–6 frames were measured with an oscillation angle ($\Delta\varphi$) of 1° using the 'constant-dose mode' of data collection. The radiation dose, size of the collimator slits and crystal-to-detector distance were the same for all test measurements and the exposure time per image ($t_{\rm exp}$) was approximately 30 s. The diffraction patterns were recorded to 1.3 Å resolution. The overall time per cycle of repetitive flash-cooling and quality checking was approximately 10 min. Owing to the high symmetry of the *R*32 space group, 5–6 frames accounted for 20–25% of the complete data set in any crystal orientation. This was sufficient for estimation of the *B* factor and $\langle I/\sigma(I)\rangle$ ratio. The number of *hkl* reflections that were partial was approximately 10 000 in each test.

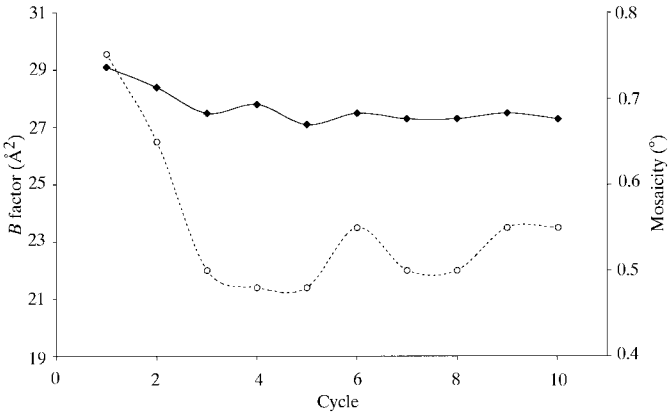

Figure 1
The effect of the number of flash-cooling cycles on the *B* factor (solid line) and mosaicity (dashed line) of the PPase crystal at constant cryosolution composition.

Subsequently, using the derived conditions for improving the diffraction quality of the PPase crystals, data sets to atomic resolution with completeness higher than 95% have been collected (Table 1). These reflection intensities were measured in two separate runs to diffraction limits of 1.05 Å ($\Delta\varphi = 0.7°$, $t_{\rm exp}$ = 6 min) and 1.8 Å ($\Delta\varphi = 1.0°$, $t_{\rm exp}$ = 15 s), in order to overcome the limitations of the dynamic range of the detector. The cut-off for the high-resolution data was set to the point where 50% of the data had $I/\sigma(I) = 2$.

3. Results

3.1. Repetitive flash-cooling with constant concentration of cryosolution components

The reversibility of cryogenic cooling and the effect of repetitive flash-cooling on the diffraction quality of the PPase crystal were studied using a cryosolution with an unchanging concentration of components. To prevent evaporation, the drop was covered between the repetitive flash-cooling cycles. The volume of the drop was 0.15 ml.

The crystal went through ten flash-cooling cycles without loss of diffraction. Variations in the unit-cell parameters during repetitive flash-cooling were within the accuracy of the measurements (0.2 Å). Changes in the diffraction quality of the crystal were observed only in the first two repetitive flash-cooling cycles (Fig. 1). The first PPase flash-cooling resulted in an increase in mosaicity (to 0.8°) compared with the crystal at ambient temperature. The resolution limit was approximately the same. A decrease in mosaicity and moderate resolution improvement was observed in the second and third cycles. From the third cycle on, there were virtually no further changes in the quality of the diffraction pattern.

A comparison of reflection intensities indicated that the molecular structure also remained unchanged from the third cycle onwards. The *R* factor of merged data for cycles 3–10 was 3.8%. The *R* factor between data sets of these cycles and the data set from the first cycle was 12%. This can be explained by

small structural changes that take place in the process of attaining equilibrium between the crystal and cryosolution in the initial flash-cooling cycles.

3.2. Repetitive flash-cooling with gradient increase in concentration of all cryosolution components

The correlation between the diffraction quality of PPase crystals and cryosolution composition was checked by changing the concentration of all cryosolution components. The cryosolution drop was left to evaporate in air. The drop volume was measured with a Hamilton GC syringe to estimate approximately the current cryosolution concentration before each flash-cooling cycle. The initial volume of the drop was 0.05 ml. Two series of measurements were made and the results are shown in Fig. 2.

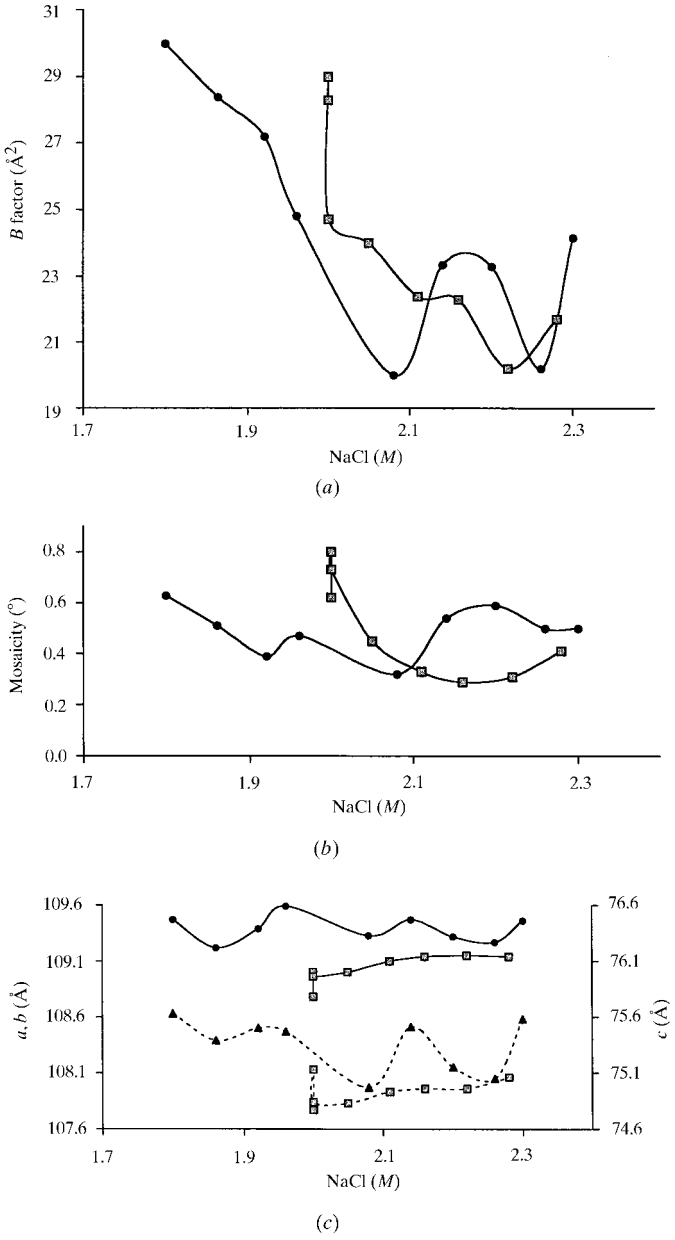

Figure 2
Repetitive flash-cooling with a gradient increase in concentration of all cryosolution components for two PPase crystals. (*a*) *B* factor, (*b*) mosaicity, (*c*) unit-cell parameters (*a*, *b* – solid lines; *c* – dashed lines) as functions of the salt concentration in the cryosolution.

During the first experiment, the crystal was placed in a cryosolution containing 1.80 *M* NaCl and 27% glycerol. As the number of flash-cooling cycles and hence the concentration increased, the quality of the diffraction patterns improved, and after the fifth cycle the *B* factor and mosaicity reached their minimum. The diffraction images after the second and the fifth cycles of flash-cooling are compared in Fig. 3. Further increase in the cryosolution concentration led to an increase of these two parameters, although the value of the Wilson *B* factor dropped again in the eighth cycle, with the mosaicity remaining essentially unchanged. The last (ninth) cycle of these measurements demonstrated an increase in the Wilson *B* factor without any change in mosaicity.

The second experiment involved crystal transfer into a cryosolution with higher initial concentrations than in the first case (2.00 *M* NaCl and 29% glycerol). The first three cycles were performed at a constant concentration of cryosolution. After the third flash-cooling cycle, the cryosolution drop was allowed to evaporate. The maximum improvement in resolution was achieved at the seventh cycle.

Both experiments confirmed that there is a correlation between the diffraction quality of the PPase crystals and an increase in the cryosolution component concentrations. The estimations of the salt concentration in the cryosolution showed that the improvement in resolution for all experiments was observed in the range 2.10–2.25 *M* NaCl. The glycerol concentration changed from 27 to 35% in case I and from 29 to 33% in case II.

3.3. Repetitive flash-cooling with increase in concentration of one of the cryosolution components

To determine which of the cryosolution components has the most pronounced effect on the diffraction quality of PPase crystals, two series of cryosolutions were prepared: one consisted of solutions with a stepwise NaCl concentration and a constant glycerol concentration, whereas the other series had a constant NaCl concentration and stepwise increases in the concentration of glycerol. The crystals were subsequently flash-cooled in solutions with increasing concentration of one of the components. The results are illustrated in Figs. 4 and 5.

The experiments with varying NaCl concentration were performed using three crystals: two of them over the range 1.86–2.25 *M* NaCl and one over the range 1.90–2.90 *M* NaCl. The behaviour of the crystals was similar in all experiments. As the NaCl concentration was increased to approximately 2.15 *M*, the diffraction pattern became progressively better. Plots of *R* factors and $\langle I/\sigma(I)\rangle$ *versus* resolution bin (Fig. 6) demonstrate that there is significant improvement in the data statistics for high-resolution reflections. A further increase in concentration led to poorer diffraction. As the concentration increased still further, the diffraction quality again improved, and at 2.80 *M* NaCl there was a pronounced second peak in order. To verify the reproducibility of these results, in the first

experimental run, after flash-cooling in cryosolution with 2.25 *M* NaCl concentration, the crystal was transferred back into the cryosolution with 2.18 *M* NaCl, followed by 2.12 *M* NaCl. This procedure was repeated in the third experimental run, where the crystal was returned to the cryosolution with 2.80 *M* NaCl concentration after the 2.90 *M* solution and twice flash-cooled.

The experiments confirm the dependence of the PPase crystal order on NaCl concentration and its independence of the number of flash-cooling cycles. The mosaicity was dependent on the crystal size, the number of flash-cooling cycles and the initial crystal quality, rather than the cryosolution concentration. In all experiments, the mosaicity after the first flash-cooling was higher than 0.7°. Improvement was observed directly after the second or third flash-cooling cycle, the mosaicity remaining virtually the same (0.3°) during subsequent cycling. The variations in the unit-cell parameters were no larger than 0.2–0.3 Å.

The experiments with varying glycerol concentration (28–38% glycerol) were performed using two crystals (Fig. 5). The measurements failed to reveal a noticeable correlation between the PPase crystal order and the glycerol content in the concentration range studied. The crystals used in these experiments were approximately half the size of those in the previous case. Owing to this fact, the mosaicity of both crystals was found to be rather low (0.2°), even after the first flash-cooling, and only slightly changed from cycle to cycle. No changes in the unit-cell parameters were detected.

3.4. Improvement of the resolution by soaking crystals to saturating salt concentrations with subsequent flash-cooling

It was noticed that PPase crystals placed in a partially evaporated drop of mother liquor prior to flash-cooling diffracted X-rays to a higher resolution than usual. To investigate this fact, we undertook the following experiment. One

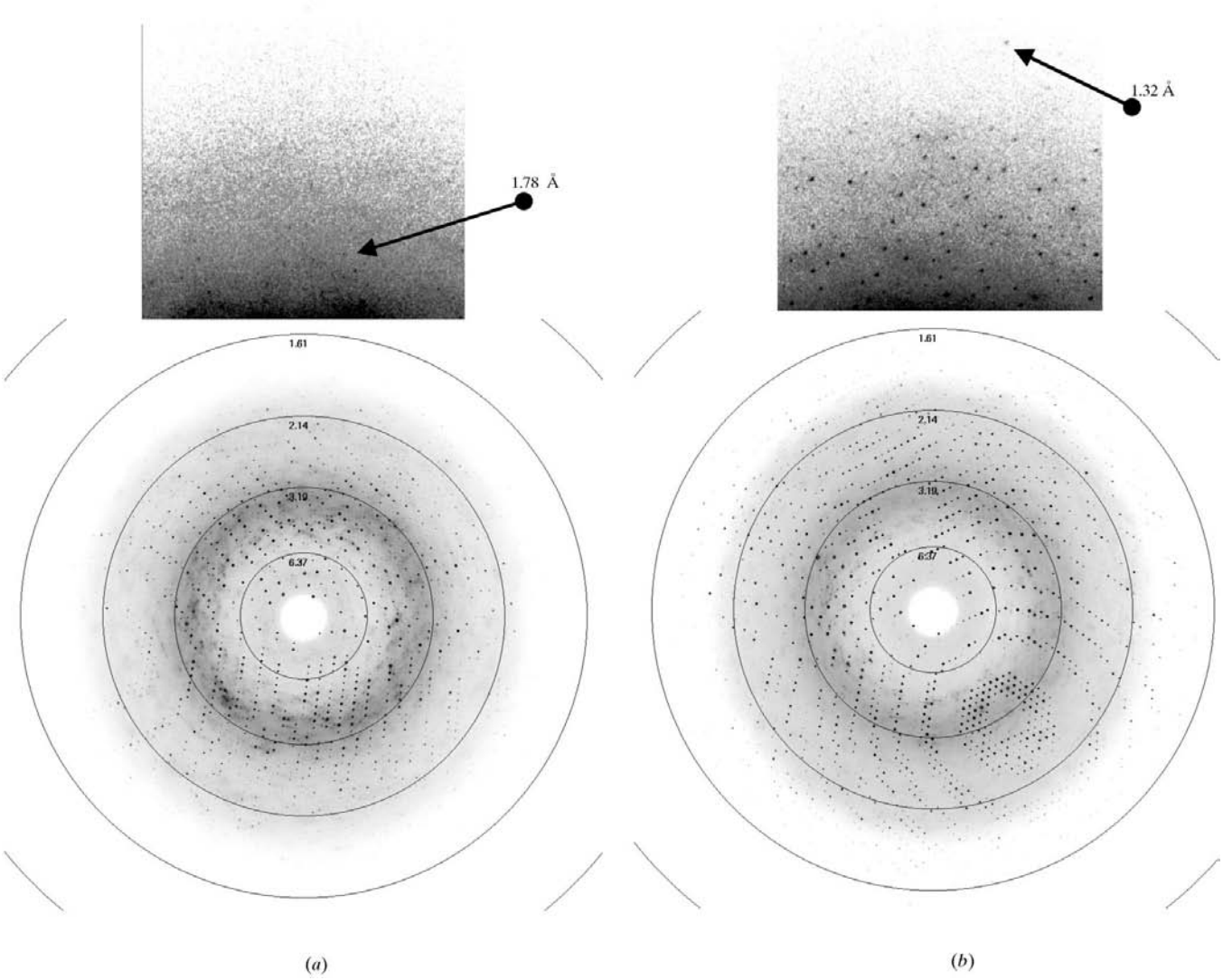

Figure 3
A comparison of diffraction from the same PPase crystal demonstrating the improvement of resolution as a result of a gradient increase in concentration of all cryosolution components. The diffraction images taken after (*a*) the second and (*b*) the fifth cycle of flash-cooling (series 1 in Fig. 2). Resolution markers are indicated. Insets show the diffraction at the high-resolution limit.

Table 2
PPase crystals at ambient temperature.

N	Unit-cell parameters (Å) a, b	c	Mosaicity (°)	Wilson B (Å^2)	Resolution (Å)	Preparation conditions
1	111.2	76.6	0.15	29.1	1.8	Conventional
2	111.2	76.2	0.12	26.0	1.6	Soaked in 2.2 *M* NaCl (about 4 h)
3	111.0	76.4	0.07	24.2	1.5	Soaked in 2.2 *M* NaCl (about 4 h)

of the PPase crystals was placed into a drop of mother liquor of 0.05 ml volume. The drop was left exposed to air for 2 h and slowly evaporated at ambient temperature. The NaCl concentration became saturated (about 5 *M* NaCl); this was indicated by the appearance of small salt crystals in the solution. At this moment, the crystal was transferred to a loop and placed into cryosolution (2.90 *M* NaCl, 30% glycerol) for no longer than 5 s followed by immediate flash-cooling. The Wilson *B* factor of this crystal was 17 Å^2 and the mosaicity was less than 0.3°. The experiment with pre-soaking until salt saturation was reached was repeated with other samples. We succeeded in achieving significant diffraction improvement several times, which resulted in data collection to resolutions of 1.2 and 1.15 Å for the PPase complexes with Ca^{2+} and Mg^{2+}, respectively (Table 1).

In the experiment involving saturating salt concentrations, the time the crystal was soaked in the cryosolution was a critical parameter. Significant improvement of the diffraction was observed only at very short soaking times (3–6 s). When the soaking time was increased to 15 s, we failed to observe a marked improvement in the diffraction pattern. The diffraction range for this crystal did not extend to further than 1.6–1.5 Å. In another experiment, a drastic change in salt concentration between the soaking solution and cryosolution accompanied by increasing the time the crystal was kept in the cryosolution to 1 min resulted in crystal destruction.

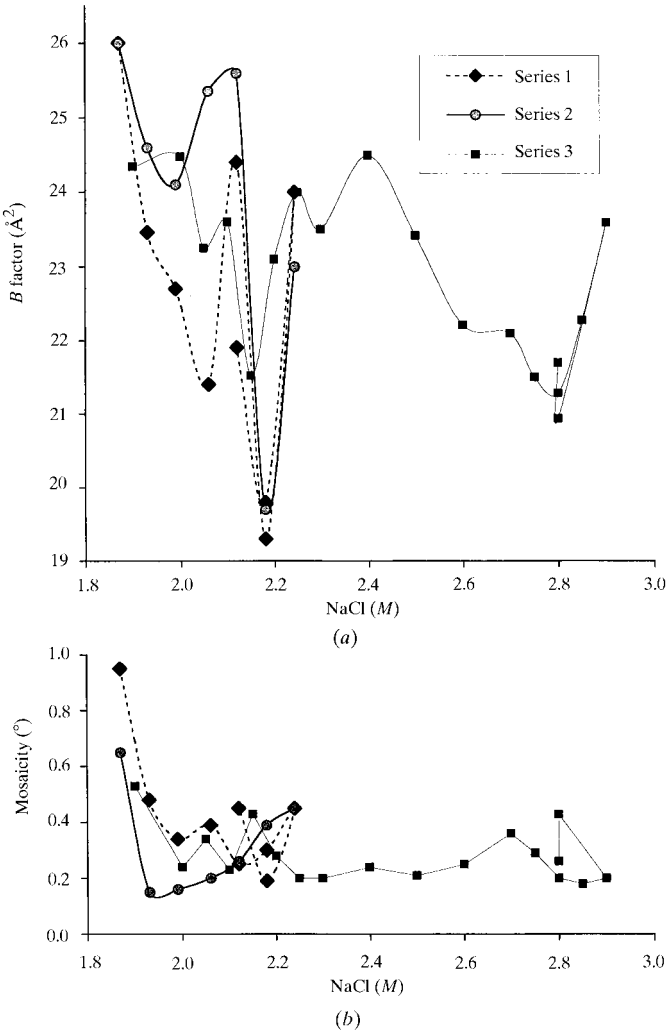

Figure 4
Repetitive flash-cooling with increase in concentration of NaCl for three PPase crystals. (*a*) *B* factor, (*b*) mosaicity as functions of salt concentration in the cryosolution.

3.5. The data obtained at room temperature

A systematic study of the effect of the component concentrations of the solutions on the diffraction quality of crystals at room temperature requires much time and large numbers of crystals. Thus, for example, the improvement in the diffraction of the MTCP-1 crystals was observed only after 1–5 months of post-crystallization soaking (Fu *et al.*, 1999). We performed two test experiments to find out the possible effect of salt concentration on the diffraction quality of PPase crystals at room temperature. Table 2 illustrates the results for one of the crystals with no change in concentration and two crystals with short (about 4 h) post-crystallization soaking in 2.20 *M* NaCl solution. In both cases, the increase in the concentration of salt led to improvement in the diffraction from 1.8 to 1.6–1.55 Å.

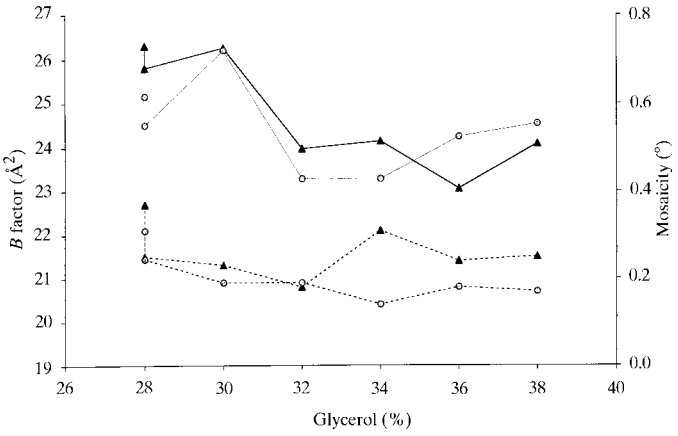

Figure 5
Repetitive flash-cooling with increase in concentration of glycerol for two PPase crystals. *B* factor (solid lines) and mosaicity (dashed lines) as functions of glycerol concentration in the cryosolution.

4. Discussion

It has been established that PPase crystals can tolerate multiple transitions from room to cryotemperature (up to ten and more times) without damage. Repeated flash-cooling beyond the first two cycles only leads to a moderate resolution improvement. The changes in molecular structure are caused by attainment of equilibrium in the bulk of the crystal.

The crystals undergo no damage with a gradual variation of cryosolution composition during flash-cooling cycles. This offers an opportunity to study the dependence of the diffraction quality of crystals on cryosolution composition for a single specimen.

The salt concentration exerts the strongest influence on the PPase crystal order. There are at least two values of NaCl concentration, approximately 2.15 and 2.80 *M*, where a significant improvement of PPase crystal order has been observed which is independent of the initial crystal quality and the method of transfer of the crystals to the optimal concentration.

The drastic effect of salt concentration on the PPase crystal order is confirmed by soaking crystals to saturating salt concentrations with subsequent flash-cooling. During such soaking, the NaCl concentration varied over a wide range, from 1.8 *M* to approximately 5 *M*. From these experiments only, it is impossible to establish whether the order is improved at saturating salt concentrations or whether the crystal undergoes ordering at another lower concentration and then remains unchanged at higher concentration.

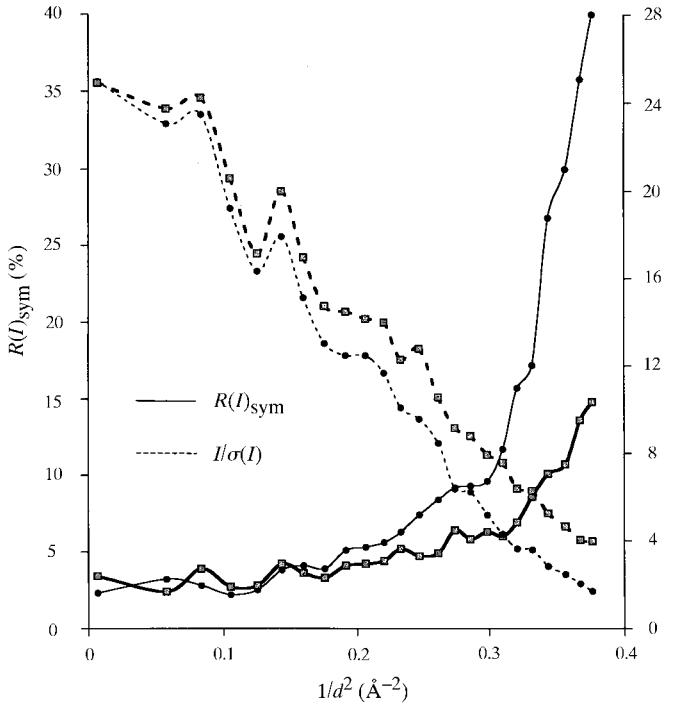

Figure 6
Data statistics for two test data collections (completeness is about 30%; redundancy, 1.5) from the same PPase crystal (series 1 in Fig. 4) flash-cooled in the cryosolution with NaCl concentrations of 1.93 *M* (thin line, *B* factor = 23.5 Å^2) and 2.18 *M* (thick line, *B* factor = 19.3 Å^2). Data were cut off at a resolution of 1.65 Å, since $\langle I/\sigma(I)\rangle$ was below 2 and $R(I)_{sym}$ was above 40% for the first data set.

Only moderate improvement in the resolution of the PPase crystals was observed at room temperature after soaking in a solution containing a salt concentration close to the optimum cryoexperiments. The most probable reason for such a minor improvement is the absence of glycerol among the solution components. It cannot be excluded that glycerol, as well as salt, plays an important role in the PPase ordering. By replacing part of the water by glycerol, we alter the salt effect on the molecular ordering in the crystal. Another possible reason may be insufficient soaking time.

It is not quite clear how quickly crystal transformations take place upon a change in concentration and what the role of flash-cooling and warming is. Soaking times in cryosolution were short and were not longer than 25 s. Nevertheless, the correlation observed in the behaviour of different PPase crystals and reproduction of the results in the process of cyclic transferral indicates that the soaking time was sufficient for the crystal to attain equilibrium with the cryosolution. It is possible that under 'flash-warming', the transformation of the crystal lattice towards equilibrium takes place very quickly. This circumstance is very important in terms of using flash-cooling cycles as a means of searching for optimal cryosolution compositions and developing a corresponding experimental protocol.

Contrary to dehydration (Esnouf *et al.*, 1998), variations in salt concentration cause small changes in the unit-cell parameters and volume within 0.3–0.4 Å and 1%, respectively (Fig. 2*c*). It was also shown that post-crystallization soaking for several days led to improvement in the diffraction accompanied by a minor (0.3%) change in the unit-cell volume of MTCP-1 crystals (Fu *et al.*, 1999).

The molecular packing in the crystal and the molecular structure depend on the salt concentration in a cryosolution. As the salt concentration increases with the number of flash-cooling cycles, the divergence between the initial and subsequent data sets increases and reaches the largest divergence for the points corresponding to the highest crystal order (series 1 and 2 in Fig. 7*a*). The *R* factor increases in all resolution shells, this being an indicator of changes in the locations of both the mobile surface parts of the molecules and the molecules of the crystal lattice (Fig. 7*b*, series 3). Comparison of data sets obtained from different specimens demonstrates a high reproducibility of the molecular structure at the same cryosolution concentrations (series 3 in Fig. 7*a*, series 1 and 2 in Fig. 7*b*).

The common observation that the mosaicity of cryocooled crystals tends to be higher than that of the same crystal at room temperature (Garman, 1999) holds also for PPase crystals. The crystal mosaicity depends weakly on the solution concentrations and crystal order; it is mainly controlled by the number of flash-cooling cycles. As the rate of crystal cooling is critically dependent on the crystal surface-to-volume ratio (Garman, 1999), the larger the crystals, the higher the mosaicity after the first flash-cooling. As has recently been shown by the analysis of diffraction patterns

from tetragonal lysozyme crystals at room temperature and at 100 K (Nave, 1998), the main contribution to broadening of rocking curves upon flash-cooling arises from variations in unit-cell dimensions between blocks rather than by increases in the angular spread of the mosaic blocks. The appearance of such imperfections can be accounted for by stress development as a result of inhomogenous freezing of different parts of a crystal. In the case of multiple flash-cooling, annealing causes the stresses either to decrease or disappear completely, thus leading to a decrease in mosaicity.

Fig. 8 presents four examples illustrating the average intensities of reflections and background as functions of resolution for the PPase crystals at ambient temperature and flash-cooled at different NaCl concentrations in the cryosolution. The background for the experiments at low temperatures is virtually the same; for room temperature, it is somewhat higher, which is accounted for by additional scattering from the capillary. Taking into consideration that the crystal volume was approximately the same in all cases, the coincidence of the low-angle average intensities of reflections indicates that the reflectivity of PPase crystals is independent of flash-cooling and concentrations of cryosolution. Therefore, the improvement of the resolution observed in the diffraction patterns can only be attributed to the change in the PPase crystal order.

While discussing the possible reasons for the diffraction improvement in MTCP-1 crystals (Fu *et al.*, 1999), it was suggested that this phenomenon can arise from rearrangement of surface residues, which form better packing interactions in the process of long-term soaking in the stabilizing solution. It was suggested previously (Sousa, 1995) that transferring crystals of proteins which contain disordered regions into solutions of glycerol or other polyols might enhance the order and structure of those regions. Adding agents such as salts, PEG and MPD to the solution in optimal proportions can lead to the same effect and finally to higher order in the crystal.

To conclude, the improvement of the PPase crystal order resulting from flash-cooling experiments combined with concentration screening allowed us to collect data sets with atomic resolution for three complexes of *E. coli* inorganic pyrophosphatase.

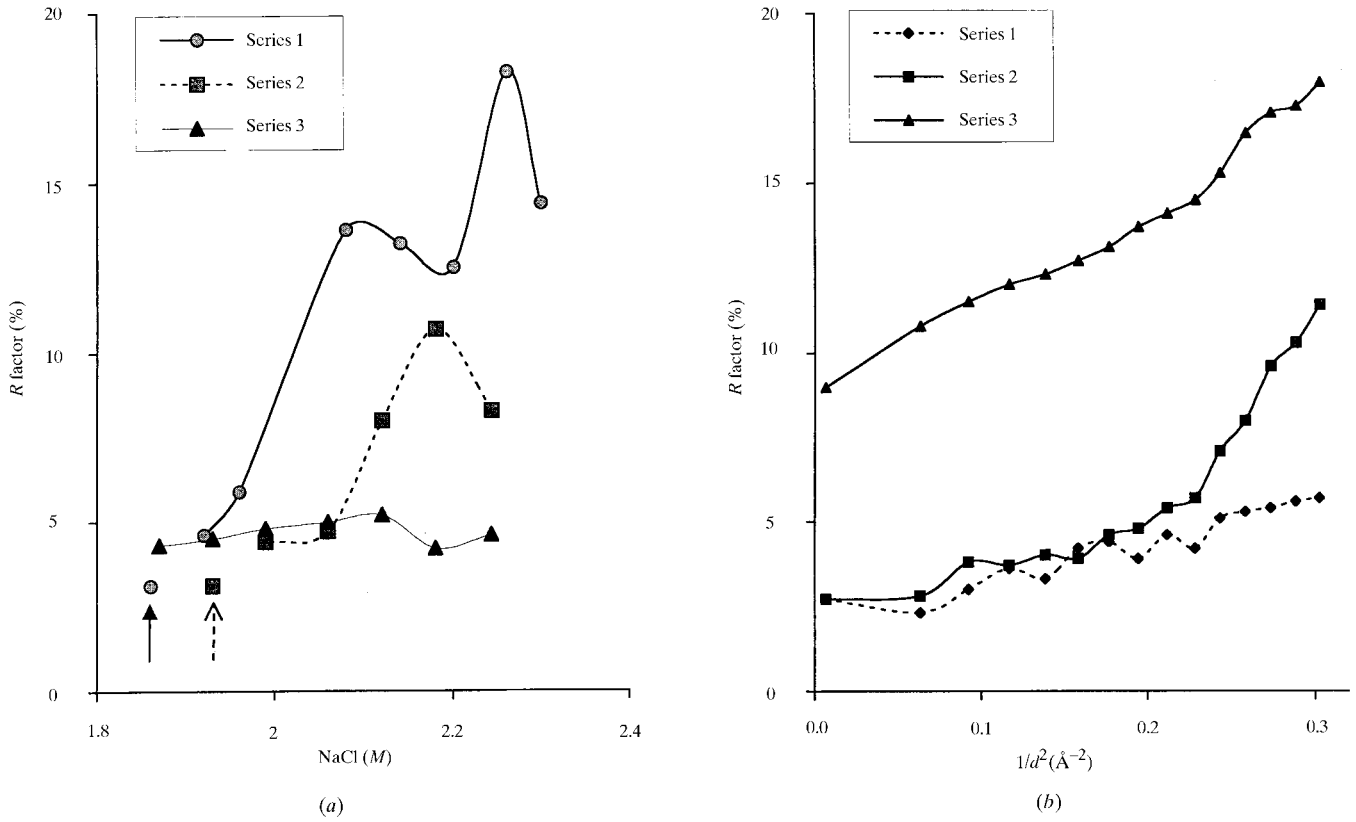

Figure 7
(*a*) Comparison of data sets. Series 1: *R* factor between the data set flash-cooled in the cryosolution with 1.86 *M* NaCl concentration (marked by an arrow and indicating the R_{merge} value when obtaining this set) and other data sets obtained with flash-cooling with concentration variation of all cryosolution components (Fig. 2). Series 2: *R* factor between the data set flash-cooled in the cryosolution with 1.93 *M* NaCl concentration (marked by an arrow and indicating the R_{merge} value when obtaining this set) and other data sets obtained with flash-cooling with only the salt concentration increasing (Fig. 4). Series 3: *R* factor between the data sets of two different specimens flash-cooled in cryosolution of the same composition (Fig. 4). For series 1 and 2, the data sets after the second flash-cooling were used in order to diminish the effects of structure transformations associated with the initial flash-cooling. To calculate the *R* factor, data in the resolution range 20.0–1.8 Å were used. (*b*) The dependence of the *R* factor on resolution. Series 1 and 2: *R* factor between the data sets of two different specimens (Fig. 4) obtained with flash-cooling in the cryosolution with the same component concentrations and NaCl concentration, 2.18 and 1.93 *M*. Series 3: *R* factor between data sets obtained with flash-cooling of the same specimen (Fig. 4) in the cryosolution with different salt concentrations (1.93 and 2.18 *M*).

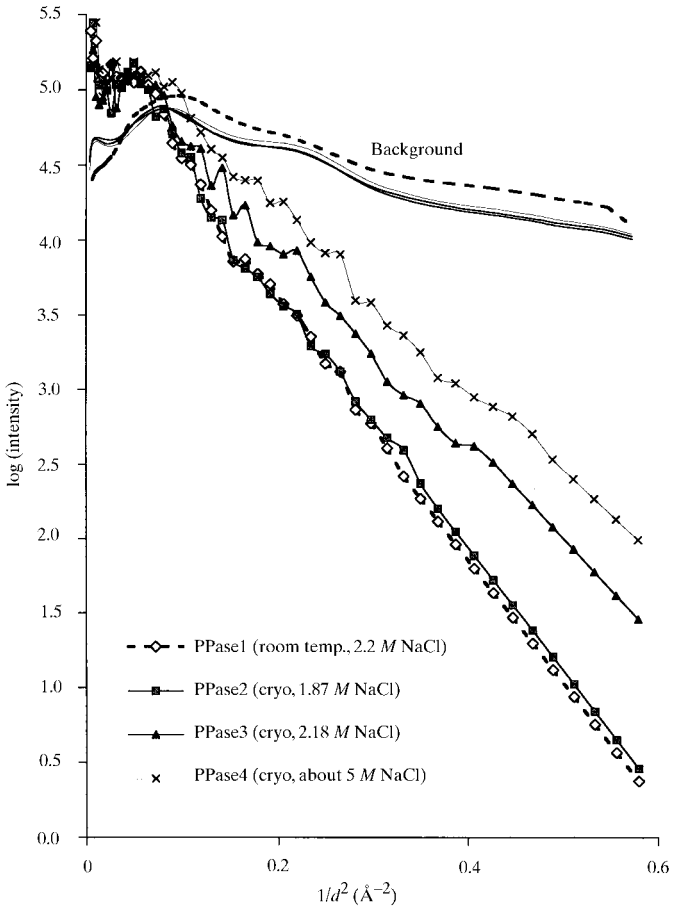

Figure 8
Resolution dependence of average reflection intensities and average background intensities under the reflection spot area for PPase crystals. PPase1: at ambient temperature after 4 h soaking in the solution with 2.2 *M* NaCl concentration (2 in Table 2). PPase2 and PPase3: the same crystal (series 2 in Fig. 4) flash-cooled in the cryosolution with NaCl concentrations 1.87 and 2.18 *M*, respectively. PPase4: flash-cooling after crystal soaking to saturating NaCl concentration. The experimental conditions for all the specimens were the same, with an exposure time of about 0.5 min. The crystal volume was approximately the same in all cases. The data on reflection intensities (without corrections for the Lorentz factor and polarization) were extracted directly from x-files after *DENZO* processing. The background intensity was obtained by averaging over circular resolution shells on diffraction images after subtracting reflection intensities. For the measurements of PPase1–3, the data on average reflection intensities at high resolution were obtained by linear extrapolation.

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