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More rapid evaluation of biomacromolecular crystals for diffraction experiments

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The parameters used to evaluate biomacromolecular crystal quality [$R_{\rm merge}$, $I/\sigma(I)$, maximum resolution and mosaicity] strongly depend on the experimental diffraction conditions. In this paper, the distinctive features of the relative Wilson plot method are described and it is shown that the overall B factor obtained from this plot is more appropriate for the characterization of protein crystals. The relative Wilson plot has been applied to the characterization of crystals of the B-DNA decamer d(CCATTAATGG) and crystals of the proteins DsrD (dissimilatory sulfite reductase D) and hen egg-white lysozyme (HEWL), which were studied by neutron diffraction. It was found that the crystal quality of the B-DNA decamer and DsrD depended significantly on the regions of the crystallization phase diagram from which the samples were taken. However, in the case of HEWL crystal quality appears to be independent of the region of the crystallization phase diagram.

1. Introduction

A simple and rapid method for the evaluation of crystal quality has not yet been established. In many protein crystallography studies, various parameters, such as the $I/\sigma(I)$ value, $R_{\rm merge}$, rocking curve, mosaicity, overall B factor and maximum resolution, have all been used at one time or another to evaluate the crystal quality. However, many of the experimental diffraction conditions affect these parameters.

The $I/\sigma(I)$ ratio is a very popular parameter for estimating crystal quality in many protein crystal-growth studies, especially cases in which the effect of microgravity on crystal quality was explored (Asano & Fujita, 1992; Koszelak *et al.*, 1995; Borisova *et al.*, 1996; Vaney *et al.*, 1996; Long *et al.*, 1996; Broutin *et al.*, 1997; Ng *et al.*, 1997; Dong *et al.*, 1999; Berisio *et al.*, 2000, 2002; Eschenburg *et al.*, 2000; Kitano *et al.*, 2000). However, one should bear in mind that the $I/\sigma(I)$ and $F/\sigma(F)$ values are very sensitive to the crystal size (see §3.3). If the $I/\sigma(I)$ value is used to estimate crystal quality, a careful normalization taking into account the crystal size should be used. However, it is difficult to measure accurately the dimensions of small crystals on a micrometre scale and crystal size normalization has not been applied in many studies (Vaney *et al.*, 1996; Broutin *et al.*, 1997; Dong *et al.*, 1999; Berisio *et al.*, 2000; Eschenburg *et al.*, 2000; Kitano *et al.*, 2000). Therefore, the $I/\sigma(I)$ parameter is difficult to use to assess the quality of the sample crystal unless normalization of the sample volume is accurately carried out.

$R_{\rm merge}$ represents the precision of the agreement between symmetry-related reflections. Thus, $R_{\rm merge}$ has been used as a parameter to assess the quality of diffraction data (Gewirth,

2001). One of the drawbacks of using $R_{\rm merge}$ as a measure of crystal quality is that this parameter depends on the accuracy of the measurements.

Currently, the most commonly used parameter to assess crystal quality appears to be the maximum resolution limit of the diffraction pattern (Koszelak *et al.*, 1995; Vaney *et al.*, 1996; Long *et al.*, 1996; Broutin *et al.*, 1997; Esposito *et al.*, 1998; Berisio *et al.*, 2000, 2002; Eschenburg *et al.*, 2000). However, strictly speaking, there are some problems with this approach as the diffraction data themselves depend on various conditions such as the intensity of the X-ray source, the diffractometer, the exposure time used, the sample-crystal size and so on. For example, if the crystal volume increases, it will be relatively easy to observe high-resolution diffraction. Moreover, even if the maximum resolution limits of the different crystals are the same, the values of the completeness of the resolution shells will depend on the researchers. On the other hand, the most important aim of our study is to assess and to select the best crystal, which is essential for high-resolution diffraction, without needing to consider the effects of the various conditions of the experiments and data analysis.

The overall *B* factor derived from a Wilson plot is the most reliable parameter for evaluating crystal quality (Asano & Fujita, 1992; Vaney *et al.*, 1996; Broutin *et al.*, 1997; Eschenburg *et al.*, 2000). There is an intimate relationship between the overall *B* factor and the limiting resolution. However, to obtain the overall *B* factor, a conventional Wilson plot typically requires information from reflections to a limit of less than 3 Å in Bragg spacing. Therefore, the measurement of high-resolution data is a prerequisite for the calculation of a meaningful Wilson plot. In order to overcome this problem, we are proposing a 'relative Wilson plot' method (see §2). The slopes of the relative Wilson plots between different crystals can lead to a quantitative measurement of the differences in the overall *B* factors. DeLucas and coworkers have discussed the effect of microgravity on the crystal quality of proteins, in which the data sets from space- and earth-grown crystals were compared using relative Wilson plots (DeLucas *et al.*, 1989). The effect of microgravity on the crystal quality of the polypeptide proteinase K has also been reported (Eschenburg *et al.*, 2000). In these papers, the space-grown crystal was used as a reference sample in the relative Wilson plots. Morimoto and coworkers also used the concept of a relative Wilson plot (Morimoto *et al.*, 1995) to discuss the relative thermal motions of three types of protein (ferredoxin, haemoglobin and superoxide dismutase) as reference samples, in which the overall *B* factors of the reference samples had been determined previously.

Generally speaking, crystallographers have aimed to grow a single crystal of sufficient size and quality for X-ray data or neutron data collection. In many cases, commercially available crystallization screening kits have been used to obtain crystals of macromolecules. However, these kits simply control the kinds of the precipitants used and the probability of success is determined by chance. In this paper, we describe the use of a 'relative Wilson plot' method to screen crystals of various proteins (obtained under different crystallization conditions) individually in order to determine the best crystallization conditions. It is a very systematic approach and we believe it is close to the ultimate goal of determining the optimum conditions for crystal growth.

We have described the crystallization phase diagram of the B-DNA decamer d(CCATTAATGG), which we have used to grow a large single crystal (1.7 × 1.3 × 0.6 mm; Arai *et al.*, 2002). A crystallization phase diagram is a very effective method of finding the boundary conditions for crystal growth. However, it is not sufficient to reveal the best conditions for crystal quality. We propose that a combination of the phase-diagram technique followed by a rational characterization of the quality of the crystals from their X-ray diffraction patterns will lead to the establishment of ideal crystallization conditions for diffraction experiments.

2. Experiments and analysis

X-ray diffraction data were collected using DIP 2020 (MacScience Inc.) and R-AXIS IV (Rigaku Co.) diffractometers. The data sets were processed with the program *Mac-DENZO* (Otwinowski & Minor, 1997) and then merged with the program *SCALEPACK* (Otwinowski & Minor, 1997). Wilson plots were calculated using the *CCP*4 set of programs (Collaborative Computational Project, Number 4, 1994).

Early in the process of determining a crystal structure, it is possible to obtain an overall *B* factor as well as a scale factor [required for putting the intensities $I_{\rm obs}(hkl)$ on an absolute scale] by calculating a Wilson plot (Blundell & Johnson, 1976),

$$Y = \left[\langle I_{\rm obs}(hkl) \rangle / \sum_j f_j^2 \right] = k \exp(-2B \sin^2\theta/\lambda^2). \quad (1)$$

(1) indicates the relationship between the experimental data $I_{\rm obs}(hkl)$, the overall temperature factor *B* and the scale factor *k*. *Y* is the intensity, which is normalized by the atomic form factor f_j, and λ is the wavelength of the incident X-ray beam (in our case, 1.5418 Å). To determine the values of *B* and *k*, the equation is rewritten in the form

$$\ln(Y) = \ln(k) - 2B(\sin^2\theta/\lambda^2). \quad (2)$$

The Wilson plot is then drawn by plotting $[\ln I_{\rm obs}(hkl)]$ *versus* $(\sin\theta/\lambda)^2$. If the *B* factor is low, then the inclination angle of the Wilson plot will be small. This implies that high-resolution diffraction spots can be observed by long exposure times to the X-ray beam. Therefore, we can regard a low *B* factor as corresponding to a good-quality crystal. However, a hump appears around d = 4 Å in the conventional Wilson plot and makes the drawing of a reliable slope difficult, especially when there is a lack of high-region data.

To solve this problem, a modified Wilson plot (which we will call a 'relative Wilson plot' in this manuscript) has been proposed according to

$$\ln\left(\frac{Y_B}{Y_A}\right) = \ln\left(\frac{k_B}{k_A}\right) - 2(B_B - B_A)\left(\frac{\sin^2\theta}{\lambda^2}\right). \quad (3)$$

(3) means that the Wilson plot of a crystal *A* (hereafter called a 'reference crystal') is used to normalize the Wilson plot of crystal *B*. Using this normalization, the hump around $d = 4$ Å in the plot disappears and the information from a wide range of scattering angles can be used. If the overall *B* factor of crystal *B* is larger than that of crystal *A*, the relative Wilson plot shows a negative slope.

In this paper, we describe our results from a double-stranded B-DNA decamer d(CCATTAATGG), from hen egg-white lysozyme (HEWL) and from the DsrD protein (dissimilatory sulfite reductase D).

3. Results and discussion

3.1. B-DNA decamer d(CCATTAATGG)

The crystallization conditions for the B-DNA decamer d(CCATTAATGG) using batch methods have been previously described using the phase-diagram technique (Arai *et al.*, 2002). Fig. 1 shows the phase diagrams for this $MgCl_2$–DNA system determined by the microbatch method, in which the drops were kept at 279 K for 20 d. The pH was kept constant at 7.0 (using 0.1 *M* sodium cacodylate buffer) and the concentrations of the precipitant MPD (2-methyl-2,4-pentanediol) were held at 31.5%(*v*/*v*) (Fig. 1*a*) and 30%(*v*/*v*) (Fig. 1*b*), respectively. The photographs in Fig. 1 show the actual single crystals used in the subsequent crystal-quality evaluation. We have found that it is difficult to quantitatively evaluate the differences between crystals using visual methods, so we have decided that an assessment of the X-ray diffraction patterns of the crystals is a more reliable technique.

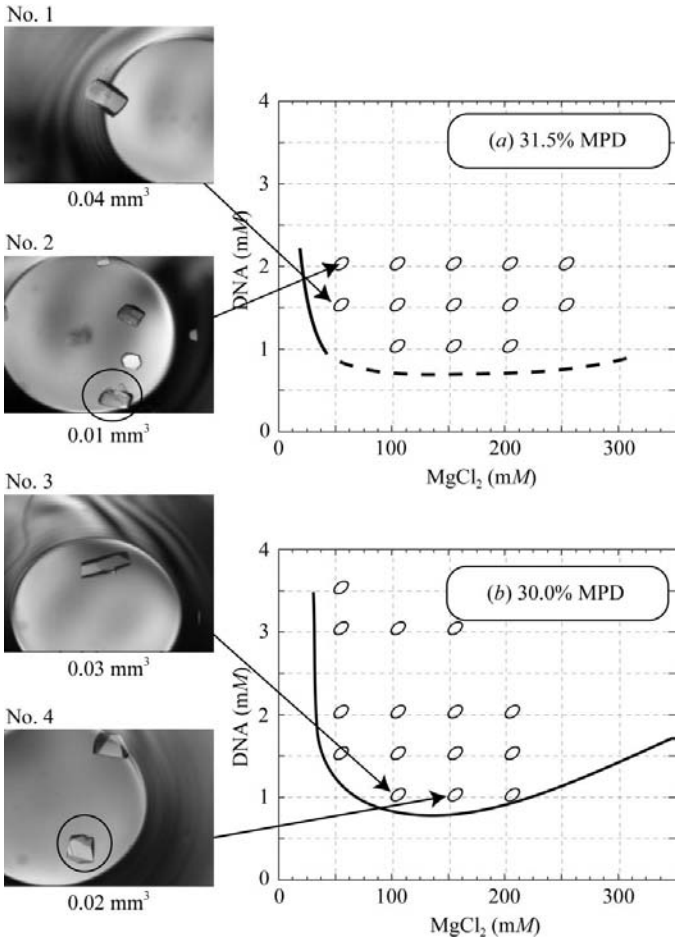

Figure 1
Crystallization phase diagrams of the B-DNA d(CCATTAATGG) under two different conditions, showing the solubility curves (crystallization boundaries) and the actual physical appearance of representative crystals.

X-ray diffraction data were collected using a DIP2020 X-ray diffractometer (MacScience Inc.) with a crystal-to-detector distance of 70 mm. The collimator used was 0.5 mm in diameter. An Oxford Cryosystems Cryostream was used to flash-cool the DNA crystals to 233 K in a nitrogen vapour stream. The oscillation angle was 2° and the total oscillation range used to obtain the Wilson plot and the relative Wilson plot was 30°.

Fig. 2 shows the conventional Wilson plots from the various DNA decamer crystals studied in our experiment. Only the plot of crystal 1 shows a wide range of scattering angles from $(\sin\theta/\lambda)^2 = 0.001$ Å^{-1} (or $d = 14$ Å) to $(\sin\theta/\lambda)^2 = 0.035$ Å^{-1} (or $d = 2.7$ Å). Moreover, the plots of crystals 2, 3 and 4 show some scatter; consequently, it is difficult to determine the overall *B* factors for those crystals directly from the plots in Fig. 2. To solve this problem, we computed relative Wilson plots by comparing the overall *B* factors obtained from different two crystals in a pairwise manner (3).

Fig. 3 shows examples of relative Wilson plots using crystal 1 as the reference sample. The differences between the overall *B* factors were derived from (3). The slope of the plot of the reference sample, namely $B_1 - B_1$, obviously equals 0. On the other hand, crystals 2, 3 and 4 show negative slopes against crystal 1 and the steepness of the slopes increases in the sequence $B_1 - B_1$, $B_3 - B_1$, $B_4 - B_1$ and $B_2 - B_1$. Thus, in this case it happens that the overall *B* factor of crystal 1 is the

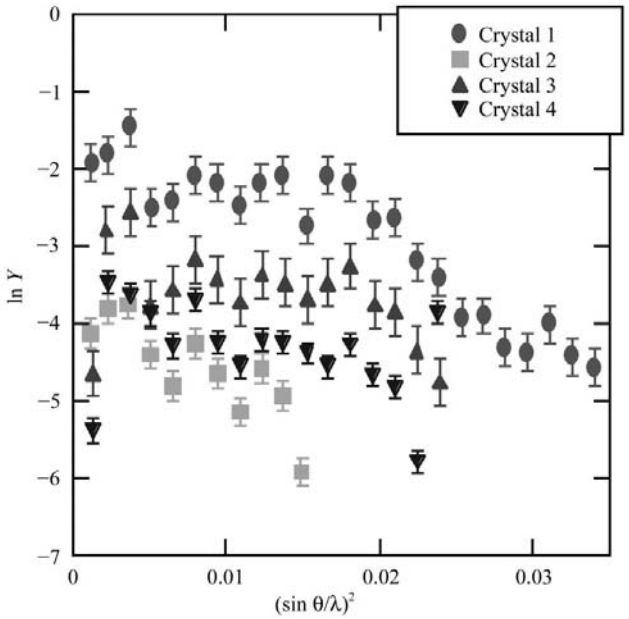

Figure 2
Wilson plots of the four different oligomer DNA crystals crystallized using different crystallization conditions.

lowest of the various crystals tested and therefore we can judge that crystal 1 is the best crystal for structural analysis. (Note: it will subsequently be shown that selection of the best crystal is not sensitive to the choice of the 'reference crystal'.) In addition, the slope of crystal 3 is very close to 0; hence, it can be presumed that the quality of crystal 3 is equivalent to that of crystal 1. We have already successfully grown a single crystal of this DNA decamer with a volume of 2.77 mm^3 using the same crystallization conditions as those for crystal 3. These results suggest that the quality of the DNA crystal improves with an increase in crystal size near the DNA solubility minimum point in Fig. 1. In other words, the quality and size of the DNA crystal depends significantly on the crystallization conditions. If the crystallization is carried out under highly supersaturated conditions far from the crystallization boundaries of the phase diagram, the molecules may become incorporated into the crystal too rapidly and the quality of the crystal will decrease as a result of this molecular disorder. Therefore, one approach to growing good crystals is to carry out the crystallization close to the solubility minimum point, as shown by points No. 1 and No. 3 in Fig. 1.

What we have shown is that even in cases in which it is difficult to obtain a complete Wilson plot (in other words, in cases where high-resolution data cannot be observed), the 'relative Wilson plot' method described here can be utilized to select a high-quality DNA crystal.

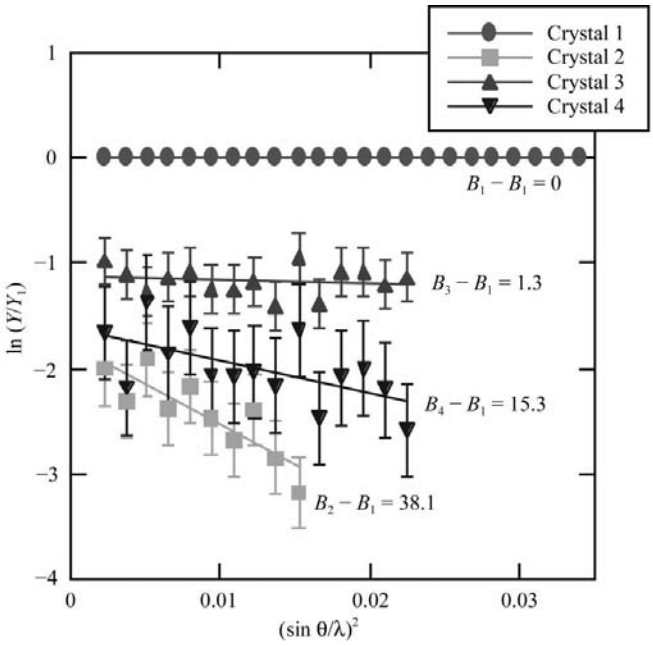

Figure 3
The relative Wilson plots corresponding to Fig. 2, in which crystal 1 is used as the reference sample.

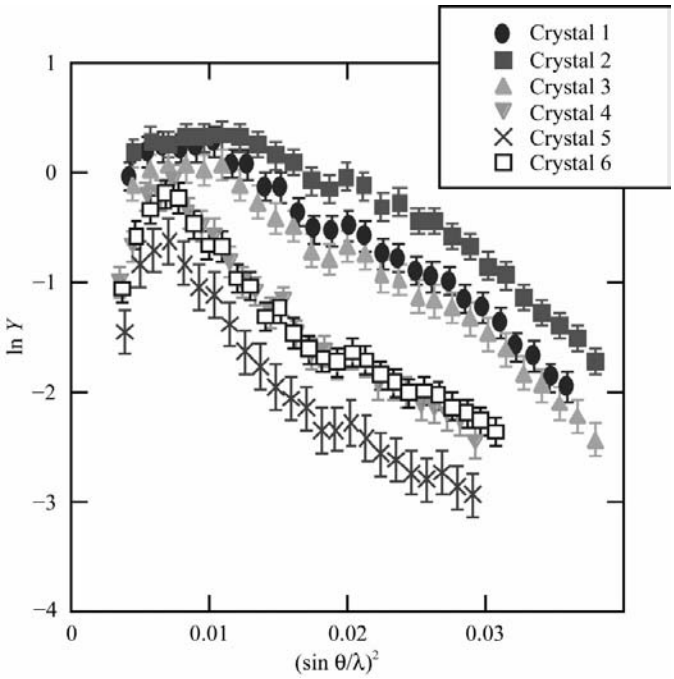

Figure 4
Wilson plots from the six different DsrD crystals crystallized using different crystallization conditions.

3.2. DsrD

DsrD (dissimilatory sulfite reductase D) is a DNA-binding protein. Recently, neutron diffraction experiments and a crystal structural analysis of this protein were carried out by our group using the BIX-3 neutron diffractometer at JAERI (Chatake *et al.*, 2003). We evaluated the crystal quality of six DsrD crystals obtained by the hanging-drop vapour-diffusion method. X-ray diffraction data were collected using a DIP2020 X-ray diffractometer (MacScience Inc.) with a crystal-to-detector distance of 75 mm. The collimator used was 0.5 mm in diameter. Fig. 4 shows the Wilson plots from these six crystals. The data from the DsrD crystals give linear plots in the range $d < 3$ Å ($\sin^2\theta/\lambda^2 > 0.028$), but their plots do not show any appreciable differences in their slopes. On the other hand, the differences in their overall B factors can be clearly distinguished by applying the 'relative Wilson plot' method.

Fig. 5 shows the relative Wilson plots of the six crystals featured in Fig. 4, using crystal 1 as the reference sample. Crystal 2 and 6 show positive slopes against crystal 1, meaning that the overall B factors of crystals 2 and 6 are lower than that

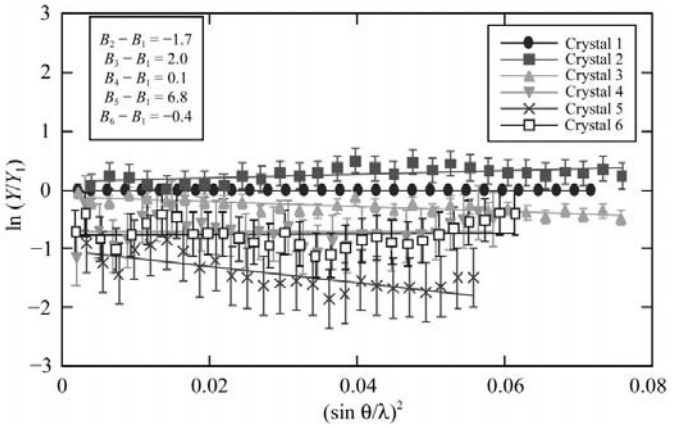

Figure 5
The relative Wilson plots corresponding to Fig. 4, in which crystal 1 is used as the reference sample.

of the reference crystal. Thus, the 'relative Wilson plot' method is not sensitive to the selection of the reference crystal and it has thus been demonstrated that this is an easily accessible method for estimation of crystal quality.

3.3. Hen egg-white lysozyme (HEWL)

Fig. 6 shows the crystallization phase diagram of HEWL determined by the microbatch method, in which the drops were kept at 293 K for two months at pH 7.0. We evaluated the crystal quality of five HEWL crystals obtained from the crystallization conditions, shown as point Nos. 1–5 in Fig. 6.

Fig. 7 shows photographic images of the actual HEWL crystals used in this analysis. In particular, note that condition No. 5 corresponds to a situation of high supersaturation, in which there were amorphous precipitates as well as crystals. X-ray diffraction data were collected using an R-AXIS IV diffractometer (Rigaku Co.) at 293 K with a crystal-to-detector distance of 80 mm. The collimator was 0.5 mm in diameter. The oscillation angle was 2° and the total oscillation ranges for data collection was 45°. Exposure times were 120 s per frame. Fig. 8 shows the Wilson plots obtained using (2). The data from the HEWL crystals gives linear plots in the range $d < 3$ Å ($\sin^2\theta/\lambda^2 > 0.028$); therefore, we could estimate the overall B factor of each crystal according to the conventional procedure described earlier. As shown in Fig. 8, the overall B factors of each crystal have almost the same values. Fig. 9 shows the relative Wilson plot in Fig. 8, in which crystal 2 is selected as the reference sample. The differences in the overall B factor between crystal 2 and the other crystals are nearly zero. These results indicate that the overall B factors of HEWL crystals do not depend on the crystallization conditions, whereas earlier it was shown that the overall B factors of DNA crystals significantly depend on the crystallization conditions. It therefore appears that the crystal quality of HEWL does not depend on the crystallization conditions.

On the other hand, it is difficult to assess the crystal quality from $I/\sigma(I)$. Fig. 10 shows a graph for four HEWL crystals in which the ratio of the intensity to error [$I/\sigma(I)$ values] are plotted as a function of resolution $(\sin\theta/\lambda)^2$. As described earlier, the experimental conditions (X-ray source, diffractometer, oscillation angles, total oscillation range, exposure times, crystal-to-detector, collimator size, temperature and so on) were normalized. However, the volumes of crystal Nos. 1, 3, 4 and 5 were 0.7, 1.1, 1.1 and 1.1 mm^3, respectively. Thus, it can be presumed that the exposed volume of crystal No. 1 was smaller than those of other crystals. The plot of crystal No. 1 in Fig. 10 differs markedly from those of other crystals, whereas the plots of the other three crystals are almost the same. In addition, as shown earlier in Figs. 8 and 9, the overall B factors of all assessed HEWL crystals were also almost the same.

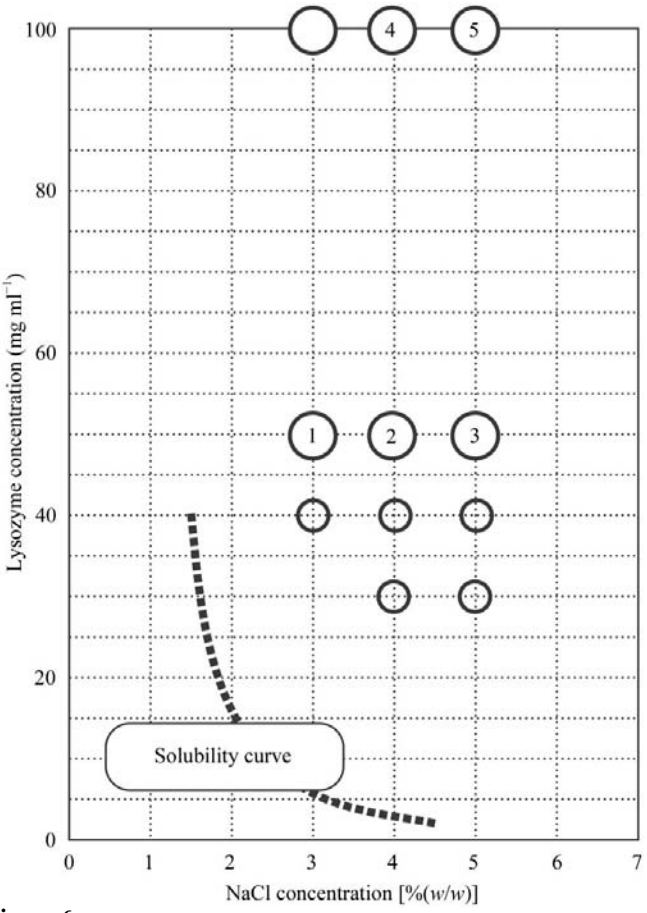

Figure 6
The phase diagram of HEWL including a solubility curve; five different crystallization conditions are indicated.

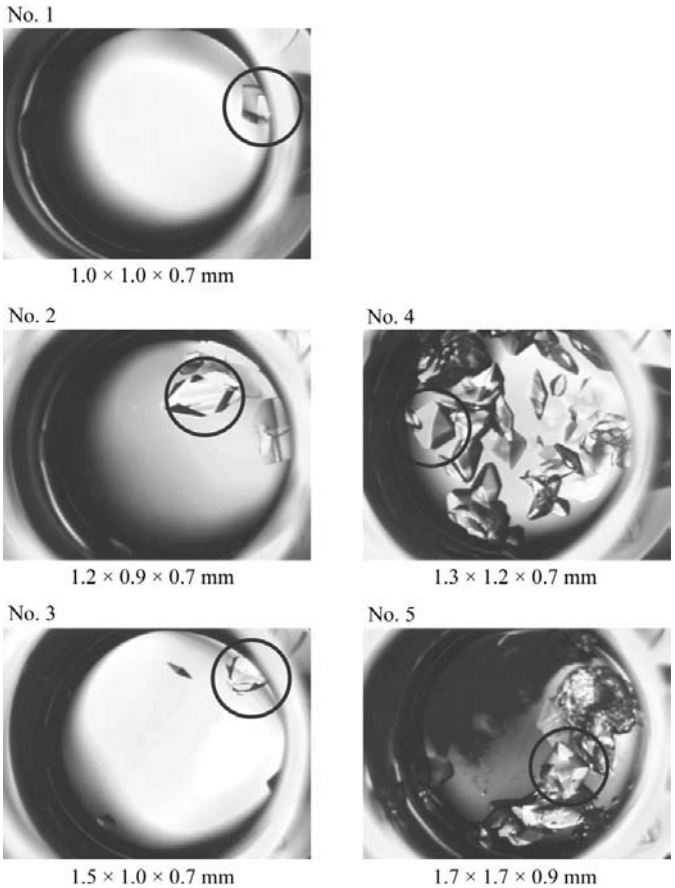

Figure 7
Five HEWL crystals crystallized under five different conditions.

Table 1
A hypothetical example showing the estimated error of $I/\sigma(I)$ affected by the error of the crystal size.

	Crystal *A*	Crystal *B*
Crystal volume (mm^3)	0.1	0.4
Diffraction intensity I	1	4
$\sigma(I) = I^{1/2}$	1	2
$I/\sigma(I)$	1	2

These results indicate that the value of $I/\sigma(I)$ is strongly affected by the crystal volume that is exposed by X-ray beam, even if the other parameters have almost the same values.

We can easily explain the reason why $I/\sigma(I)$ is not an adequate parameter to assess crystal quality, as indicated by a hypothetical example shown in Table 1. If we assume that the qualities of crystal *A* and crystal *B* are the same, then we should expect the same $I/\sigma(I)$ values for both crystals *A* and *B*. If the ratio of the crystal sizes is 1:4, the ratio of the reflection intensities will also be 1:4 because the reflection intensity is proportional to the crystal volume. However, the ratio of $\sigma(I)$ values will be 1:2, since $\sigma(I)$ is estimated by $\sigma(I) = I^{1/2}$. Therefore, the ratio of $I/\sigma(I)$ will be 1:2. Thus, these values of $I/\sigma(I)$ are in conflict with the first assumption that the crystal qualities of the crystal *A* and *B* are same. When we use $I/\sigma(I)$ values to estimate crystal quality, a careful normalization of crystal size is required. However, crystal size has not usually been normalized in most studies (Asano & Fujita, 1992; Koszelak *et al.*, 1995; Borisova *et al.*, 1996; Vaney *et al.*, 1996; Long *et al.*, 1996; Broutin *et al.*, 1997; Ng *et al.*, 1997; Dong *et al.*, 1999; Berisio *et al.*, 2000).

The propagation of error should also be taken into account when the $I/\sigma(I)$ value is discussed. If the x represents one dimension of the crystal, the crystal volume will be roughly estimated as x^3. Since the reflection intensity is proportional to the crystal volume, it will also be proportional to x^3. $\sigma(I)$ is then estimated as $x^{3/2}$ and $I/\sigma(I)$ can be estimated as $(x^3)/(x^{3/2}) = x^{3/2}$. This indicates that the error in the $I/\sigma(I)$ value is about the 3/2 power of the error in the measurement of one dimension of the crystal size. For example, if the measurement for crystal size is accompanied by a 10% error in each

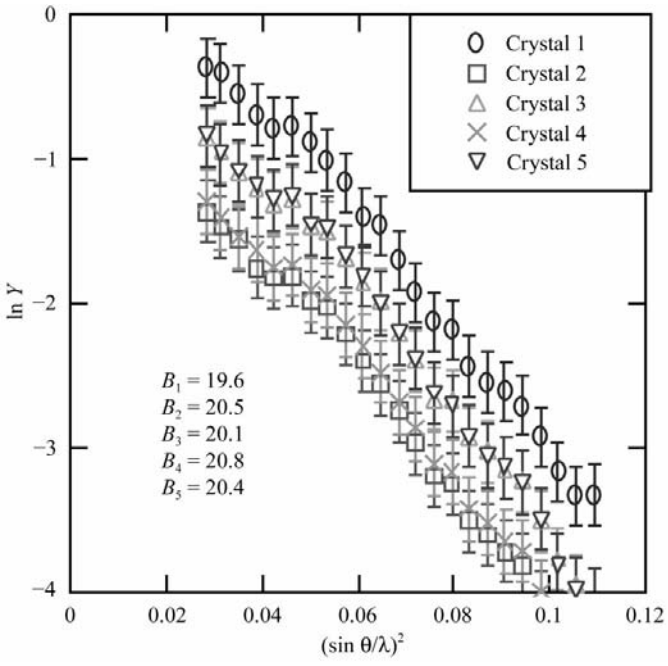

Figure 8
Wilson plots from five different HEWL crystals crystallized using different crystallization conditions.

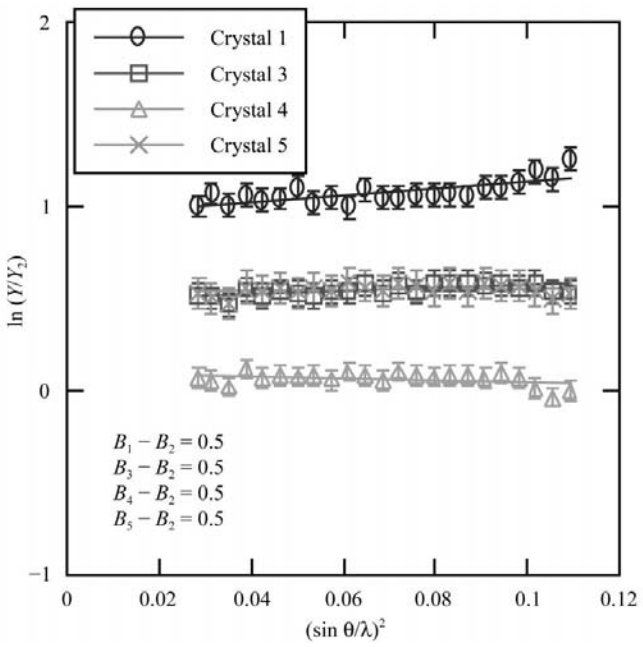

Figure 9
The relative Wilson plots corresponding to Fig. 8, in which crystal 2 is used as the reference sample.

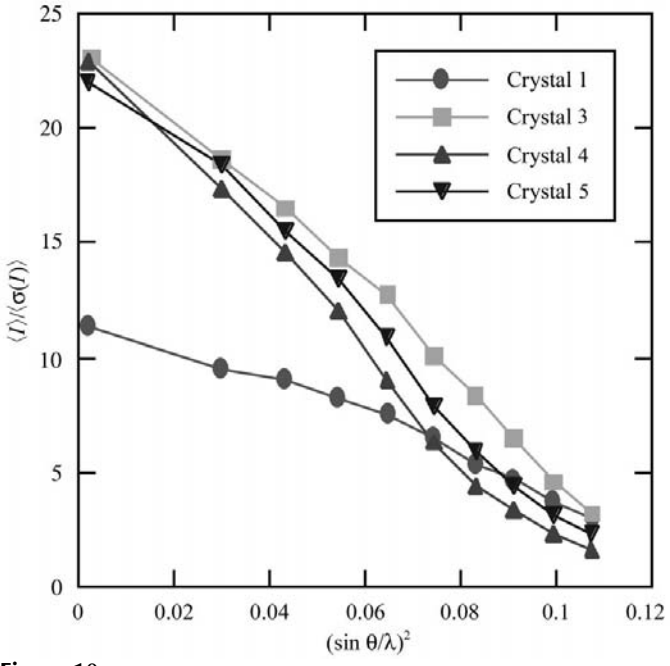

Figure 10
A plot of $\langle I\rangle/\langle\sigma(I)\rangle$ as a function of resolution ($\sin^2\theta/\lambda^2$) for HEWL crystals, using X-ray diffraction data collected from the crystals shown in Fig. 7.

dimension, it will lead to a 15% error in $I/\sigma(I)$. In many studies, crystal qualities have been compared on the basis of differences in $I/\sigma(I)$ of 10–20%. Nevertheless, it is difficult to determine the size of the crystal within 10% error in each dimension, especially when researchers deal with the extra-small samples typically used in synchrotron-radiation experiments. Therefore, it is difficult to assess the crystal quality using $I/\sigma(I)$ values. If the crystal sizes are exactly the same, $I/\sigma(I)$ can be applied to estimate the crystal quality, but in practice it will be nearly impossible to perform this precisely. It is necessary to establish a method which is not affected by experimental conditions. We think that the relative Wilson plot method described here is a rational method of assessing the crystal quality because it is less affected by various other conditions.

4. Conclusion

From the present results, we can make the following comments on the crystal growth of the biomacromolecules we have investigated.

(i) The quality and size of DNA crystals depend significantly on their crystallization conditions.

(ii) The quality of DsrD crystals also depends on their crystallization conditions. However, this tendency is not as clear as the case of the DNA decamer.

(iii) The crystal qualities of HEWL change very little in the large region of crystallization conditions that we investigated in our phase diagram (HEWL–NaCl system at pH 7.0). This result indicates that HEWL is an exceptional case compared with the two other biomolecules studied in this paper, *i.e.* DNA and DsrD.

Since crystal quality appears to depend significantly on the species of biomolecule being studied (as pointed out above), we believe that a rational method to estimate the crystal quality is required. The 'relative Wilson plot' method described here is suitable for the evaluation of crystal quality. We summarize the advantages of this method as follows.

(i) It is not affected by many of the experimental diffraction conditions (such as the X-ray source, diffractometer, exposure time, crystal volume, beam size and so on).

(ii) It can be used to estimate crystal quality using relatively small amounts of X-ray data. (For example, in the case of the DNA crystal, the total oscillating range was 30° and only about 1200 reflections were necessary to calculate a relative Wilson plot.) Moreover, only a low-resolution data set (>4 Å) is required.

Owing to these advantages, the overall B factor, which can be obtained either from a conventional Wilson plot or from a relative Wilson plot, is a more suitable parameter for rapid screening of crystal quality than the other parameters and plots mentioned in this article.

The phase diagrams and relative Wilson plots shown in this paper provide guidelines for the crystallization of biomacromolecules with high quality. Firstly, the phase-diagram technique gives a rough estimate of the regions to select for good-quality crystals (specifically, those grown near the crystallization boundary should be first selected). Then, after a small number of crystals near the solubility curve have been collected and assessed *via* their X-ray diffraction patterns, the relative Wilson plot method can be used to assess the best conditions to use to obtain a good-quality crystal. By a combination of the two techniques, we have succeeded in obtaining good-quality and large crystals (2.77 mm^3) of the B-DNA decamer d(CCATTAATGG) that are good enough for neutron data collection. Recently, the collection of a complete data set of Bragg reflections by neutron diffraction was successful and we are currently analyzing the detailed structure of that DNA duplex including the hydration pattern and the hydrogen positions.

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