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The Ultimate Wavelength for Protein Crystallography?

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Abstract

This paper describes an analysis of the optimum choice of the X-ray wavelength for macromolecular diffraction data collection. It is shown that there is no ultimate X-ray wavelength for protein crystallography and that the optimum wavelength depends to a large extent on the size of the protein crystal. It also depends on instrumental factors, such as efficiency of the detector for a particular wavelength and spectral density of the synchrotron radiation. Estimates of the optimum wavelength as a function of crystal size are given.

1. Introduction

The question 'What is the best wavelength for protein crystallography?' has recently been addressed in a number of papers (Arndt, 1984; Helliwell, 1992; Helliwell, Ealick, Doing, Irving & Szebenyi, 1993; Gonzalez & Nave, 1994; Gonzalez, Denny & Nave,1994; Nave, 1995). Some of the authors argue that the ultimate way to collect ideal data for macromolecular crystallography is to use very short (λ = 0.5 Å) and ultra-short (λ = 0.3 Å) wavelengths (Arndt, 1984; Helliwell, 1992; Helliwell, Ealick, Doing, Irving & Szebenyi, 1993). This would reduce crystal absorption errors, improve the signal-to-noise ratio and increase the lifetime of the protein crystal. As a consequence, unprecedented data quality could be achieved. Experiments, however, have shown that there is no improvement in the signal-to-background ratio for synchrotron diffraction data collected using a very short wavelength (λ = 0.55 Å) compared with data collected with λ = 0.92 Å (Gonzalez, Denny & Nave, 1994). Furthermore, although there is some evidence of an increase in the lifetime of a protein crystal during diffraction data collection at 0.9 Å compared with the lifetime when λ = 1.5 Å, there is no evidence that better quality data can be collected at even shorter wavelengths. It is not known if an increase in the lifetime of protein crystals is a general result and if this can be linearly extrapolated to the shortest wavelength available. Use of a longer wavelength (*e.g.* 2.5 Å) has been suggested for synchrotron data collection from microcrystals (Helliwell, 1993).

We undertook theoretical studies of the lifetime of protein crystals when subjected to the process of synchrotron diffraction data collection. Results suggest that there is no ultimate wavelength for protein crystallography. The choice of the wavelength depends to a large extent on the size of the protein crystal exposed to the synchrotron radiation. In most cases, 0.9 Å X-rays are 'hard' enough to minimize absorption errors and to optimize efficiency of diffraction. Further reduction of the wavelength will not improve the efficiency of diffraction experiments. It may even lead to a deterioration of the quality of the data because of a decrease of both the detector efficiency and the spectral density of the synchrotron radiation. The present study suggests that when only small crystals are available, it would be beneficial to use longer wavelengths up to 1.6–1.8 Å, or even longer. Even with crystals of larger size, it might be advantageous to select carefully 'softer' radiation for use in the diffraction experiments. Crystals as large as 0.4 mm might easily tolerate X-rays of wavelength 1.3 Å, instead of 0.9 Å. This by itself will decrease the time of exposure by a factor of two, which might be particularly beneficial for data collection at the bending-magnet beamlines of the synchrotron sources at Daresbury Laboratory, LURE, Stanford, Japan Photon Factory and Brazilian National Synchrotron Laboratory. The critical wavelength of these rings does not exceed 2.6 Å and the spectral density of the emitted radiation is significantly higher at 1.3 Å than at 0.9 Å. This will further shorten exposure times and decrease radiation damage to crystals by free radicals. We also present herein a plot of the longest recommended wavelength for use with a particular size of crystal exposed to synchrotron radiation.

2. Theoretical background

In protein crystallography, one would like to maximize the integrated intensities of the X-rays diffracted by a macromolecular crystal in order to obtain better counting statistics for the diffraction data. At the same time, the total X-ray energy absorbed by the crystal should be kept to a minimum, as this is the primary source of radiation damage and degradation of the diffraction data. In other words, we are interested in the optimization of the ratio between the integrated diffraction intensities from a protein crystal and the energy of X-ray photons absorbed

in the diffraction process. We shall refer to this ratio as 'diffraction efficiency' (Polikarpov, 1997).

In the first-order approximation, the diffraction efficiency is written as (Polikarpov, 1997),

$$(P/E_{abs}) \propto [\lambda^3 t \exp(-\mu t)]/[1 - \exp(-\mu t)]. \quad (1)$$

One can see from (1) and the relation $\mu = a\lambda^3$ that the diffraction efficiency (P/E_{abs}) is a function of two parameters: the size of the crystal and the wavelength of the X-rays. Here $a \simeq 0.22$ mm^{-1} Å^{-3} (Arndt, 1984).

Diffraction efficiency as a function of the size of the crystal has only a trivial maximum at $t \rightarrow 0$. For very small crystals there is no absorption, but there is no diffraction as well; therefore, this solution has no physical significance.

When considered as a function of the wavelength, (P/E_{abs}) reaches a maximum at very short wavelengths ($\lambda \rightarrow 0$). This agrees with the results of Arndt (1984), Helliwell (1992) and Helliwell, Ealick, Doing, Irving & Szebenyi (1993). However, the behaviour of the function will depend to a great extent on the value of the second parameter, namely the size of the crystal. The wavelength dependence of the diffraction efficiency normalized to 100% at the short-wavelength limit for three different values of the parameter t is shown in Fig. 1.

For all three crystal sizes shown in Fig. 1, the diffraction efficiency is equal to 100% at very short wavelengths. For the medium-sized crystal ($t \simeq 0.2$ mm), the diffraction efficiency decreases very slowly as the X-ray wavelength increases. At 2.5 Å, the diffraction efficiency still maintains about 75% of its initial value. The smaller crystals will show even less wavelength dependence of the parameter (P/E_{abs}). For larger crystals, approaching the size of half a millimetre, the diffraction efficiency decreases more rapidly. It reaches the 75% level at about 1.8 Å and at 2.5 Å it is less than 50%. For even larger crystals (t = 1 mm), diffraction efficiency decreases faster: it reaches 75% at about 1.4 Å and at 2.5 Å it practically vanishes. As one can see from Fig. 1, there is practically no difference in diffraction efficiency between wavelengths of 0.9 and 0.5 Å for the crystals of size 0.1–1 mm.

This functional behaviour is not surprising. The integrated diffraction intensity for a thin crystal ($t \ll \mu^{-1}$) initially grows as λ^2 increases up to the point when absorption of X-ray quanta inside the crystal becomes significant. The effects of absorption will then restrict any subsequent growth of the integrated intensity. Further increase of the wavelength of the X-rays will lead to the situation found for X-ray diffraction in a thick crystal ($t \gg \mu^{-1}$), where most of the X-ray quanta will be absorbed before exiting the bulk of the protein crystal and integrated diffraction intensities will drop exponentially. This is when diffraction efficiency rapidly decreases.

The maximum of the integrated X-ray diffraction intensities can be determined by differentiation of the expression for the integrated intensities of X-rays diffracted by a mosaic crystal with respect to λ. The result of this calculation gives (Rosenbaum & Holmes, 1980),

$$\lambda = (2/3at)^{1/3}. \quad (2)$$

Equation (2) sets a limit for the longest wavelength to be used in protein crystallographic studies. The wavelengths calculated using this formula (Fig. 2) correspond approximately to the 75% level of diffraction efficiency. It could be argued that the 75% threshold is not sufficiently high and, maybe, a somewhat higher optimum value should be chosen. However, it is certainly undesirable to use wavelengths longer than the limit specified by (2).

Both Fig. 1 and Fig. 2 indicate that 0.9 Å is a sufficiently short X-ray wavelength for most of the cases. Even for t = 1.5 mm, which is rather exceptional in protein crystallography, the critical wavelength determined by (2) is 1.25 Å, still longer than the wavelength

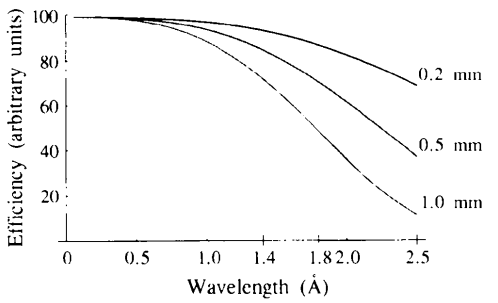

Fig. 1. Wavelength dependence of the diffraction efficiency [equation (1)] normalized to 100% at the short-wavelength limit for three different thicknesses t of the crystal. The maximum optimum wavelengths, 1.4, 1.8 and 2.5 Å, for the crystal sizes, 1, 0.5 and 0.2 mm, respectively, are indicated [see equation (2)].

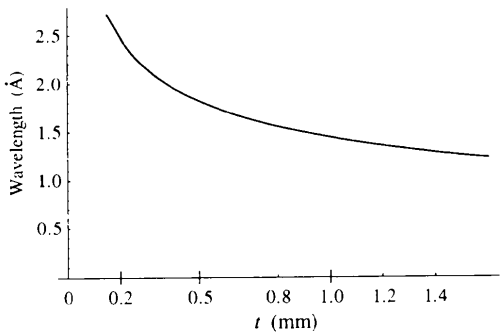

Fig. 2. The long-wavelength limit as a function of the size of the crystal, computed according to equation (2). The wavelengths plotted here correspond to approximately the 75% level of diffraction efficiency. The sizes t = 0.2, 0.5 and 1.0 mm presented in Fig. 1 are indicated.

of 0.9 Å conventionally used in protein crystallography with synchrotron radiation.

Choosing a wavelength below the limit set by (2) implies that accuracy can be satisfactorily preserved because the crystal size will be smaller than the absorption length of the X-rays and consequently it would not be necessary to apply corrections for X-ray absorption. If large (0.8–1.2 mm) protein crystals happen to grow, the lucky protein crystallographer might routinely collect data at the rotating anode, being unaware of the fact that conditions of low diffraction efficiency are prevalent (see Fig. 1). It is also probable that no absorption corrections would be applied and, therefore, the accuracy would be compromized, even though reasonable precision could be achieved. It is wiser to use synchrotron data collection at short wavelengths for such crystals, because absorption errors will adversely influence the quality of the diffraction data.

3. Instrumental factors

The longest possible lifetime of protein crystals during data collection is not the most important goal by itself. In fact, one usually aims to collect the best possible diffraction data set in terms of statistics, signal-to-noise ratio and absorption errors, optimizing precision and accuracy. All previous considerations were based on the assumption that the spectral density of the incident radiation and the efficiency of the X-ray detector do not vary with the wavelength. This is of course not true. Therefore, instead of diffraction efficiency (P/E_{abs}) we should maximize a function $(P/E_{abs})S(\lambda)D(\lambda)$, where $S(\lambda)$ is the spectral density of the synchrotron radiation and $D(\lambda)$ is the efficiency of the X-ray detector. Both functions $S(\lambda)$ and $D(\lambda)$ can vary considerably for different beamlines and detectors. There are, however, some general rules. The spectral density of the synchrotron radiation drops rapidly beyond the critical wavelength of the beamline, at the shorter wavelength side. The critical wavelength at beamline I-5AD (SPEAR, Standford) is 2.6 Å; at D41 and D23 (LURE, DCI, Paris) it is 3.4 Å; at 7.2 (SRS-HBL, Daresbury) it is 3.9 Å; at 6 A2 (Photon Factory, Japan) it is 3.1 Å; at X-12C (NSLS, Brookhaven Biology Department) it is 3 Å (Helliwell, 1992). The best possible statistics will be achieved with a wavelength close to the critical wavelength for a particular beamline provided that equation (2) still holds. This wavelength will also shorten the time of data acquisition and decrease the damage to the crystal by free radicals created by absorbed X-rays. The penalty for choosing the shorter wavelength will be a significant increase of exposure time. For example, at the beamline 7.2 (Daresbury) a change in the wavelength from 1.488 to 1.2 Å leads to a threefold decrease in the intensity of the incident radiation (Lindley, 1995). This, in turn, will proportionally increase the exposure time.

The efficiency of detector systems is determined by the absorption of X-rays in the active area of the detector. For softer X-rays the efficiency is limited by absorption in the entrance window. This can be optimized so that wavelengths between 1 and 2 Å can be detected with nearly 100% efficiency. Below 1 Å, the efficiency of a detector might decrease exponentially, in the same way that absorption decreases. For example, at 0.5 Å, the efficiency of the image plate is only 50% and drops further at shorter wavelengths. In this situation, half of the quanta diffracted by the crystal will pass undetected by the detector, although having made a significant contribution to free-radical formation.

Naturally, as far as instrumental factors are concerned, each experimental set-up has to be analysed in order to completely optimize the choice of wavelength. Factors such as the ability to record all data on a detector of specified aperture at a single-detector setting, the effects of obliquity, the time course of the development of radiation damage, the availability and feasibility of cryo-cooling techniques for each particular crystal, and the extent to which damage is dose dependent and time dependent, will also come into play, in addition to the factors considered above.

4. Conclusions

The theoretical considerations discussed above show that there is no advantage to be gained from the use of very short and ultra-short wavelengths in protein crystallography data collection. In most of the practical cases, a wavelength of 0.9 Å is sufficiently short to minimize radiation damage to the macromolecular crystals.

For a given size of the crystal, there is a wavelength threshold above which the number of X-ray quanta absorbed in the crystal increases significantly; this threshold should not be exceeded if possible [see (2) and Figs. 1 and 2].

Although integrated diffraction intensities slightly decrease for longer X-ray wavelengths because of absorption, the total counting statistics will depend on the flux of the synchrotron radiation illuminating the sample. Unnecessary reduction of the wavelength will lead to increased exposure times and, as a consequence, the time of data collection and the effects of radiation damage will be increased. On bending-magnet or wiggler beamlines when the chosen wavelength falls on the slope of the spectral density of the synchrotron radiation, the use of shorter wavelengths might result in a considerable increase of exposure time. This will lead to unnecessary secondary-radiation damage because of free-radical formation. On the other hand, in many cases the wavelength of the X-rays used in protein crystallography with synchrotron radiation could be increased to improve the intensity of diffraction from protein crystals. This is particularly true for small and badly diffracting crystals.

A change in wavelength from 0.9 to 1.3 Å will shorten the exposure time two times and a change from 0.9 to 1.6 Å will reduce the exposure time three times, although providing the same statistics for the diffraction data. Possible improvement in the flux of the synchrotron radiation at a longer wavelength will further shorten the time needed to achieve the same quality of data collection. The wavelengths 1.3–1.6 Å are still short enough to permit data collection with correctable absorption error from crystals up to 0.6 mm in size. Improvement in the statistics of a protein diffraction data set could also result in an increase of the resolution of diffraction in cases where this was limited because of statistical reasons.

We believe that the frequently observed improvement in the lifetime of protein crystals with the change of the X-ray wavelength from 1.54 to 0.9 Å might be due to the fact that the crystals were too big, at least in one dimension, for absorptionless data collection at 1.54 Å. If the optical path of the X-rays inside the crystal exceeds 0.8 mm, in at least one of the orientations of the crystal, (2) does not hold and unnecessary absorption of X-rays will occur for $\lambda = 1.54$ Å. This will not only decrease the integrated diffraction intensities, but also create many more free radicals.

A possible experimental evaluation of the considerations above can be designed easily. One could grow a large number of identical crystals from the same batch, *e.g.* of lysozyme or trypsin, and then use crystals of similar sizes for data collections at different wavelengths. The exposure dose should be chosen carefully, from a set of, say, three frames taken from the same rotation range but with different doses; from these, the dose that will provide data at the last fixed resolution shell with the same quality should be chosen [say, 80% of the reflections in the last shell should have $I \geq 3\sigma(I)$]. With this dose, one should then collect a full data set with similar data redundancy. The final statistics from all the experiments would indicate the optimal choice of wavelength for a particular size of crystal, implicitly taking into account the experimental dependence on the critical wavelength of the source and efficiency of the detector used. A feasible experiment could be performed with three different crystal sizes (*e.g.* 0.1, 0.4 and 0.7 mm average dimensions) and at three different wavelengths (say, 0.7, 1.1 and 1.6 Å).

Synchrotron diffraction data collection making use of radiation with wavelengths in the range 1.3–1.6 Å, or even longer, is particularly important for low-energy electron rings such as that at the Brazilian National Synchrotron Laboratory (a recently constructed facility which is currently the only synchrotron source in Latin America). This facility operates at an electron energy of 1.37 GeV and has a nominal current of 100 mA. The synchrotron radiation flux from the bending magnets of the ring drops significantly at wavelengths shorter than 1.2 Å, making the use of short-wavelength radiation rather difficult. Theoretical studies presented here show the feasibility of the use of 1.3–1.6 Å synchrotron light for macromolecular crystallographic studies. We expect to be able to commence relevant experimental studies during the second half of 1997, when the protein crystallography beamline will be fully commissioned.

The use of soft X-rays, with a wavelength of 2.5 Å, in crystallographic studies of microcrystals (*e.g.* 20 μm size) has been suggested (Helliwell, 1993). The microcrystals will suffer only 9% absorption at a wavelength of 2.5 Å, but an 8.8 times increase of diffracted-beam energy will be gained with respect to that at a wavelength of 0.9 Å. Averaged normalized diffraction efficiency (Polikarpov, 1997) will be fairly low in this situation and protein microcrystals, even after cryo-cooling, will suffer from a high radiation load. However, since microcrystals are an important problem to be further addressed, lower energy synchrotron sources might be used to pursue this idea, given that they are capable of offering an equivalent flux to that provided at a shorter wavelength by higher energy bending-magnet or multipole-wiggler synchrotron radiation sources.

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