Protein microcrystal diffraction and the effects of radiation damage with ultra-high-flux synchrotron radiation

(British Prototherapy/Laue diffraction)

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ABSTRACT By using ultra-high-flux synchrotron x-radiation from a wiggler source, good Laue diffraction data have been obtained at room temperature from microcrystals of gramicidin A, a sample volume that is ≈2 orders of magnitude smaller than was hitherto possible for this type of study. The feasibility of obtaining diffraction data from microcrystals of stable, inorganic compounds of high scattering power using synchrotron radiation has been shown (5, 6).

As part of our feasibility study with the white beam to provide the high flux levels at the sample, we have also investigated the effect of radiation damage to both the microcrystals of gramicidin A as well as to normal-sized samples of hemoglobin crystals. At the maximum level of 10^{11} to 10^{14} photons per sec/mm^2 at the sample, a large quantity of diffraction data could be collected, which would represent a complete data set in the case of a higher symmetry space group or a large fraction of reciprocal space for a lower symmetry case. We conclude, therefore, that a reduction of sample volume of 2–3 orders of magnitude compared with that used in conventional synchrotron x-ray diffraction "data collection" (7, 8) represents a major advance.

Application of modified Laue diffraction δλ/λ ≈ 0.3 and full white beam Laue techniques to proteins has been demonstrated on normal-sized crystals (ref. 9; unpublished results). A successful crystal structure analysis using white beam protein Laue diffraction data, however, awaits the development of new approaches to, and software for, the extraction of structure factor amplitudes from the intensities. Ultra-high x-ray brightness electron storage rings of the next generation like the European Synchrotron Radiation Facility will provide monochromatic flux levels at the sample of the order of 10^{13} to 10^{14} or more photons per sec/mm^2 and will avoid the complication of analyzing white beam patterns from (≈20 μm)^3 samples. Obviously, white beam Laue diffraction patterns recorded with radiation from insertion devices on these future "high-brilliance" machines may allow the study of even smaller samples and remove the most intransigent

Abbreviations: DESY, Deutsches Elektronen-Synchrotron in Hamburg, Germany; SRS, Synchrotron Radiation Source in Daresbury, United Kingdom; SSSL, Stanford Synchrotron Radiation Laboratory in Stanford, California.

A number of discussions and workshops have been held in Europe and the U.S.A. concerning the design and construction of a new generation of high-brightness storage rings. In Europe, there are many reports concerning the proposed 5- to 6-GeV ring, but a new comprehensive description—"The European Synchrotron Radiation Facility," edited by B. Buras and S. Tazzari—has just been released (1984) by the European Synchrotron Radiation Project (c/o European Center for Nuclear Research, Geneva, Switzerland). Reports in the U.S.A. are found in the Proceedings of the New Rings Workshop held at Stanford University (summer of 1983) and in the report of the Major Materials Facilities Committee of the National Academy of Sciences (1984).
obstacle to the determination of biological structure at the molecular level, that of obtaining large single crystals.

METHODS AND RESULTS

The measurements were performed at the SRS protein crystallography beam line 9.6 with the storage ring running in multibunch mode at 1.8 GeV, ~300 mA, and with the superconducting wiggler operating at 4.50 T (λ ~ 1 Å). The only elements interposed between the wiggler source and the sample were several beryllium windows in the beam line, a short air path, and sometimes also a helium path (described below). The flux level intercepted by the sample is for these conditions \( \approx 10^{13} \text{ photons per sec/mm}^2 \). For comparison, the Cu Kα emission line of a 1.6-kW rotating anode gives \( \approx 10^9 \) photons per sec/mm\(^2\) at the sample. The spectral range in the beam was 0.2 Å ≤ λ ≤ 4 Å. The size of the incident beam was defined by motorized slits to \( \approx (100 \, \mu\text{m})^2 \) for the gramicidin experiments and to \( \approx (200 \, \mu\text{m})^2 \) for the hemoglobin ones. The cross-fire angles of the beam are in the horizontal ≤1 mrad and are negligible in the vertical. The Laue diffraction patterns were recorded on an Arndt-Wonaccott rotation camera using photographic film (CEA Reflex 25).

For the gramicidin experiments, crystals were initially grown under conditions published elsewhere (10) and were selected and mounted at the appropriate size. Ordinary Lindemann glass x-ray capillaries were used (0.2 mm in diameter; 0.01 mm in wall thickness). For crystals of \( \approx 20 \, \mu\text{m}^3 \) a polarizing attachment was crucial to enable the observation in the mounting stereomicroscope. The position in the capillary of the microcrystal was marked by a felt-tip pen to aid visibility in the oscillation camera telescope eyepiece on the beam line. To reduce and control the air scatter of the white beam, a helium path (an aluminium cone with two thin windows) was placed between the microcrystal and the film. Some problems were encountered in finding a suitable material for the first window, which would not interact with the white beam; eventually, a very thin (12 µm) piece of polyethylene terephthalate (tradename, Hostaphan; Hoechst) was used. This cone with the Hostaphan window was essential to get good signal-to-noise in the diffraction patterns from the microcrystals.

Fig. 1 shows a Laue diffraction pattern from the smallest gramicidin crystal used \((30 \times 35 \times 10 \, \mu\text{m}^3)\); the exposure time was 50 sec. The high quality of the pattern and the reflection intensity signal-to-noise is evident. However, this particular crystal does have a large mosaic spread and, in any case, the Laue method is more sensitive to crystal sample imperfections than monochromatic methods. As a result, the spots are smeared out and have some degree of texture. Quite clearly, a smaller sample volume could have been used from the point of view of the strength of the pattern. There is a theoretical limit to the smallest tractable sample size of \( \approx 100 \) unit cells \((\approx 1.0 \, \mu\text{m}, \text{assuming a unit cell of 100 Å}) \). This limit is due to the dispersion of interference submaxima into the regions between diffraction spots. Even a 100-cell crystal will have at least 10% of each spot intensity in these tail regions. To recover these overlapped data would require a deconvolution procedure. Hence, 1000 cells, \( \approx 10 \) µm with a 100-Å cell or 3 µm with the gramicidin cell size, is probably a more realistic limit to the smallest possible size. However, the limitation for the present experiment was the invisibility of the crystal in the available microscope.

The scattering efficiency of the crystal for the gramicidin unit cell case, expressed as \( V_s f^2 / V \) (where \( V_s \) = sample volume, \( f = \text{atomic number, and } V = \text{unit cell volume} \), is \( \approx 2.2 \times 10^{12} \text{ Å}^2 \)). This value should be compared with the values of \( 17 \times 10^{12} \text{ Å}^2 \) quoted for a 6-µm CaF\(_2\) crystal in the feasibility test performed at the Deutsches Elektronen-Synchrotron in Hamburg, Germany (DESY) (5), and of 52 ×
protein crystal data set is collectable from a standard-sized protein crystal with a flux level of $10^{13}-10^{14}$ photons per sec/mm$^2$ in <1 min of total exposure time and before major sample radiation damage occurs. The control experiment, in which the longer wavelengths in the beam were rejected, was performed to evaluate whether the radiation damage effect was ameliorated without them, the argument being that the increasingly large fraction of absorbed long $\lambda$ photons would primarily cause the damage. However, a visual inspection of the patterns produced did not reveal the expected enhancement in sample lifetime.

Finally, it is necessary to explore whether radiation damage is more severe as sample size is reduced and, therefore, how much data can be collected before decay is significant. With the gramicidin crystals described earlier, a sample of size $100 \times 20 \times 20 \mu$m$^3$ showed negligible damage by the time a complete sweep of reciprocal space had been effected ($180^\circ$ for this monoclinic case). For the ($\approx 20 \mu$m)$^3$ samples, however, only six good exposures could be collected ($\approx 90^\circ$ of reciprocal space).

In conclusion, we have demonstrated that (i) good signal-to-noise reflection intensity data can be collected from a ($\approx 20 \mu$m)$^3$ protein crystal mounted in the usual way, wet in a sealed capillary, (ii) radiation damage to such a sample is at a level that still allows a large survey of reciprocal space to be made, and (iii) for a normal-sized protein crystal a complete survey of reciprocal space is feasible in <1 min before major damage occurs. Finally, other new sources of ultra-high-intensity synchrotron radiation are now becoming available—e.g., the 54-pole wiggler at SSRL, where flux levels in the white beam, at the sample, will reach the order of $10^{15}-10^{16}$ photons per sec/mm$^2$. In the near future, this source (and flux level) can be evaluated as a tool in protein crystallography and other diffraction experiments.

**Fig. 2.** Laue diffraction patterns recorded from a standard-sized crystal of hemoglobin. (A) First film. (B) Sixth film for identical exposure time (7.5 sec) and sample orientation. The radiation damage is clearly visible in the elongation of spots and diffuseness of the diffraction pattern. (C) A computer prediction of the pattern to aid in visual inspection.
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