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Applications of synchrotron radiation to protein crystallography: Preliminary results

(x-ray diffraction/anomalous dispersion/rubredoxin/azurin/nerve growth factor/glutaminase-asparaginase)

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ABSTRACT **X-ray diffraction photographs of protein single crystals have been obtained using synchrotron radiation produced by an electron-positron storage ring. The diffracted intensities observed with this unconventional source are a factor of at least 60 greater than those obtained with a sealed x-ray tube using the same crystal and instrumental parameters. Diffraction data have been collected by the precession method to higher resolution and using smaller protein crystals than would have been possible with a conventional source. The crystal decay rate in the synchrotron beam for several proteins appears to be substantially less than that observed with Ni-filtered Cu radiation. The tunable nature of the source (which allows selective optimization of anomalous contributions to the scattering factors) and the low angular divergence of the beam make the source very useful for single crystal protein diffraction studies.**

The use of synchrotron radiation as a source for single crystal x-ray diffraction studies has recently been the subject of considerable discussion and controversy. In contrast to conventional sources, the polychromatic radiation produced by synchrotron emission can be monochromated over a large range of wavelengths to give an intense x-ray beam. Questions have been raised about the actual gain in diffracted intensity which is possible over more conventional sources and the ability of biological materials (such as protein crystals) to withstand the high radiation flux.

Synchrotron radiation has already been used for low-angle diffraction studies of biological materials. Experiments conducted at Hamburg using the synchrotron DESY have demonstrated that such radiation provides over 50 times more intensity in low angle diffraction from muscle fibers (1, 2). Some low angle diffraction results have also been reported using the VEEP-3 storage ring at Novosibirsk (3). Although a number of authors, most notably Wyckoff (4) and Harrison (5), have theoretically discussed diffraction from single crystals of macromolecules using synchrotron radiation, to date no such experiments have actually been reported.

Diffraction photographs obtained from single protein crystals with synchrotron radiation are presented in this preliminary report. We describe striking evidence that the tunability of the synchrotron source, combined with large gains in actual diffracted intensities from single crystals and surprisingly low rates of crystal damage, offers unique advantages for the use of this unconventional source in x-ray diffraction studies of protein crystals.

EXPERIMENTAL METHODS AND RESULTS

Synchrotron Radiation Source. All experiments described in this report were conducted at the Stanford Synchrotron Radiation Project, a national facility for the exploitation of synchrotron radiation produced by the SPEAR electron-positron storage ring at the Stanford Linear Accelerator Center, Stanford, Calif.

Synchrotron radiation is emitted tangentially to the instantaneous path of the charged particles as they are maintained in circular orbits around the ring by bending magnets. During this process, the particles lose energy (a continuum extending from the infrared into the x-ray region). This lost energy is restored by radio frequency accelerator cavities. The emission is confined by relativistic effects to a narrow cone with low angular divergence in the plane of the ring. At present, 4.2 GeV is the maximum particle energy for a single stored beam (electrons or positrons) and 100 mA is the maximum current.

The current slowly decays with time and the storage ring has to be periodically refilled. A typical cycle consists of 30 min to fill the ring with two beams at an energy of 1.5 GeV and raise (ramp) the energy to about 3.7 GeV, 15 min of orbit adjustments to collide the electron and positron beams, and then 2–3 hr of slowly but steadily decreasing current. The beam decays with a half life of about 2 hr, resulting in an approximate 2-fold decrease in intensity over the same period. At 3.7 GeV, there is a high flux of x-rays from 0.5 to 2.5 Å. The characteristics of the synchrotron radiation generated by SPEAR as a function of energy have been tabulated elsewhere (6).

Monochromator and Diffraction Camera. The monochromator used in these experiments was designed and constructed by a group from the California Institute of Technology. A detailed description of this crystal-mirror monochromator system and its characteristics is being published†.

The beam, which is reflected at grazing incidence from the mirror and then diffracted and focused from a Si[1,1,1] monochromator crystal, enters the experimental area (called a hutch) which is separated from the primary beam area by lead shutters. The doors of the hutch are key-interlocked to prevent access to the experimental area while the main shutters are open. Immediate access to the hutch can readily be accomplished, even though there is still beam from the storage ring, by simply closing the hutch shutters.

An Enraf–Nonius precession camera was located in the hutch to record the diffraction photographs with a monochromator to sample distance of 50 cm. In order to avoid extensive camera modification, standard pinhole collimators were used to restrict the final beam size. In future experiments utilization of guard slits is planned in order to facilitate location of the intense part of the beam. As the geometric features of collimation of the synchrotron beam have not

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† N. G. Webb, R. M. Stroud, S. Samson, R. Gamble, and J. D. Baldeschwieler (1975). Manuscript submitted.

yet been determined precisely, the intensity gain may be underestimated. Part of the reason for this effect, to be described in a later publication, is that any reduction in aperture by the collimator may decrease the effective source size and reduce the maximum possible intensity.

Camera alignment was achieved by using Polaroid film exposures to observe both the passage of the beam through the collimator and out the camera back, and to monitor the shape of the x-ray beam. For future use, an alignment system consisting of ion chambers to detect the beam and motors to remotely move the camera is being constructed. Air scattering between the shutters and the camera was minimized by inserting a helium-filled tube.

For certain diffraction experiments, it was desirable to use a wavelength close to the absorption edge of a particular metal present in the molecule. To locate a given wavelength, the beam intensity was measured by an ionization chamber, both with and without a thin foil (25 μm) of the metal interposed in the beam. By repeating this measurement at several wavelengths, the absorption edge could be detected by the abrupt decrease in intensity on the high energy side of the absorption edge. It was necessary to place the foil about 30 cm from the detector, otherwise the effect was obscured by fluorescence. This method was used to locate the Cu (1.38 Å) and Fe (1.74 Å) K absorption edges. For the proteins, the edges are expected to be shifted to slightly higher energies.

The actual wavelength was determined by recording precession photographs of a very small, strongly diffracting crystal of $SrAlF_5$ and comparing the distances between identical reflections with those on a film taken with Ni-filtered radiation from a standard copper fine focus tube and processed in the same manner. Wavelength calibrations obtained in this manner should be accurate to about ± 0.07% (7).

Crystal Diffraction Data. The experiments described in this paper were done during three periods of running at the Stanford Synchrotron Radiation Project, totalling 11 days. Precession photographs were obtained using crystals of several proteins at a number of different wavelengths (see Table 1). The crystals of rubredoxin and azurin were prepared by L. C. Sieker in the laboratory of L. H. Jensen, while those of nerve growth factor and L-glutaminase-asparaginase were grown in our laboratory.

(*A*) Rubredoxin. Rubredoxin is an iron-containing protein whose x-ray structure has been determined and refined at a resolution of 1.5 Å (8). The space group is R3 with cell constants a = 64.5 and c = 32.7 Å. The very good quality of the diffraction pattern, high stability in the x-ray beam, and the presence of one iron atom per 6000 molecular weight suggested rubredoxin as a good choice for a test protein. It could be used both for checking the feasibility of data collection and for attempts to locate the iron by the measurement of anomalous differences. Eight precession photographs of the non-centric *hkO* projection were taken at a resolution of up to 1.7 Å (see Fig. 1) with wavelengths ranging from 1.74 Å to 1.80 Å (both sides of the Fe K absorption edge).

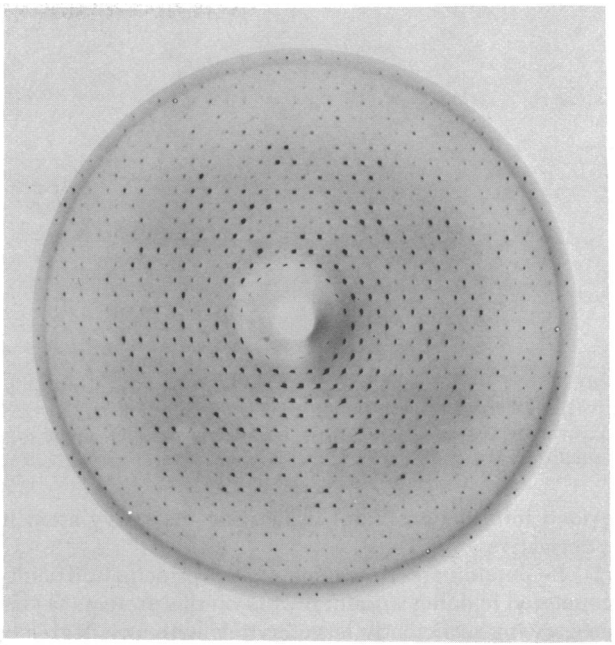

FIG. 1. Precession photograph of the *hkO* plane of a rubredoxin crystal. Synchrotron radiation, E = 3.7 GeV, average current 19 mA, λ = 1.744 Å, precession angle μ = 25°, exposure time 5 hr.

(*B*) Azurin. The structure of azurin (9), a small, copper-containing protein, has been solved to low resolution (L. H. Jensen, personal communication). The space group is $P2_12_12_1$ with cell constants a = 58.9, b = 79.0, and c = 108.5 Å. A native data set extending to 2.7 Å resolution was collected at the wavelength of 1.376 Å (just below the Cu K absorption edge) using the synchrotron source. These measurements were carried out using two small crystals (one of the crystals used was 0.4 × 0.35 × 0.12 mm and the other, 0.35 × 0.15 × 0.1 mm). The 14 useful precession films were collected on these crystals with a combined exposure time of about 28 hr.

(*C*) Nerve growth factor. A small (0.3 mm long and 0.1 mm thick) crystal of the mersalyl derivative of nerve growth factor (10) was photographed. The crystals belong to space group $P6_122$ with a = 56.1 and c = 181.4 Å. Previous attempts to obtain good quality diffraction patterns using similar crystals and an Elliott 2 kW rotating anode generator were unsuccessful. However, using the synchrotron source, a 5-hr exposure resulted in a photograph (Fig. 2) which has

Table 1. Summary of diffraction data obtained with synchrotron radiation

Protein	Number of precession films	Resolution, Å	Plane	Wavelength, Å	Exposure time, hr	Crystal size, mm
Rubredoxin	8	1.7–3	*hkO*	1.74–1.80	2–5	0.5 × 0.4 × 0.3
Azurin	14	2.7	3-D	1.376	1.5–2.5	0.4 × 0.35 × 0.12 0.3 × 0.15 × 0.1
Nerve growth factor	1	6.0	*hOl*	1.74	5	0.3 × 0.1 × 0.1
L-Glutaminase-asparaginase	1	3.5	*hOl*	1.376	2	0.5 × 0.05 × 0.05

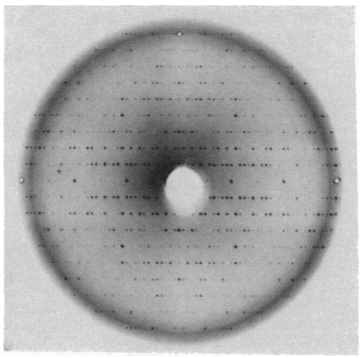

FIG. 2. Precession photograph of the *hOl* plane of the mersalyl derivative of nerve growth factor. Synchrotron radiation, E = 3.7 GeV, average current 21 mA, λ = 1.740 Å, precession angle μ = 10°, exposure time 5 hr.

provided information useful in locating the heavy atom in this derivative.

(*D*) L-Glutaminase-asparaginase. The principal difficulty encountered in doing structural work on this protein (11) has been growing sufficiently large crystals with any degree of reproducibility. A 3.5 Å resolution photograph (space group $P6_2$, a = 145.0, c = 63.5 Å) of the *hOl* plane was obtained in 2 hr using a needle-shaped crystal, 0.5 mm long and only 0.05 mm thick. While its quality is still not exceptionally good, it clearly shows that, if the crystal is advanced through the beam in order to minimize radiation damage, useful data can be obtained on these small crystals.

DISCUSSION OF RESULTS

Intensity and Crystal Stability. The exposure times of photographs taken with the synchrotron source (Table 1) were dramatically shorter than exposure times necessary to obtain photographs with the same crystals and a conventional sealed tube. It was possible to routinely collect high angle precession photographs in 1.5–2.5 hr of exposure time. A cone-axis oscillation (12) photograph of azurin obtained in 10 min at 3.7 GeV and 40 mA, using a 0.5 mm collimator is shown on the left in Fig. 3. The photograph is more intense and clearly extends to higher resolution than the photograph reproduced to the right in Fig. 3, which was obtained using the same crystal, collimator, and camera settings with a 6-hr exposure using a Philips fine focus x-ray tube operated at 40 kV, 30 mA. Intensities were measured (from the cone axis films shown in Fig. 3) by scanning densitometry for 20 pairs of reflections which could be indexed as being equivalent. The average background-corrected intensity on the synchrotron film was slightly higher (a few percent) than that of the film taken with the conventional tube. Thus there is a minimum gain in diffracted intensity of 60-fold for the synchrotron source (this lower limit being imposed simply by the exposure times). Similar results were subsequently obtained with precession photographs. Similar comparisons with the photographs taken on an Elliott rotating anode generator operated at 40 kV, 40 mA (300 μm × 3 mm focus) with Ni-filtered CuKα radiation indicate at least a 6-fold intensity gain for the synchrotron source. One of the most powerful rotating anodes used for protein crystallography is the 12 kW Rigaku with a 0.5 × 10.0 mm focal spot. For this unit, the gain in flux over the Elliott mentioned above is calculated to be 2.7, thus the intensity from the synchrotron source is still substantially higher. The intensities available at noncharacteristic wavelengths are orders of magnitude higher than from any other available source. In these experiments, it has been possible to utilize photon energies within 20 eV of the absorption edges of copper and iron where, using a conventional source, there would not be sufficient intensity to be useful. It should be emphasized that the experimental arrangements used in these experiments were not optimized and that collimator and alignment modifications in the more

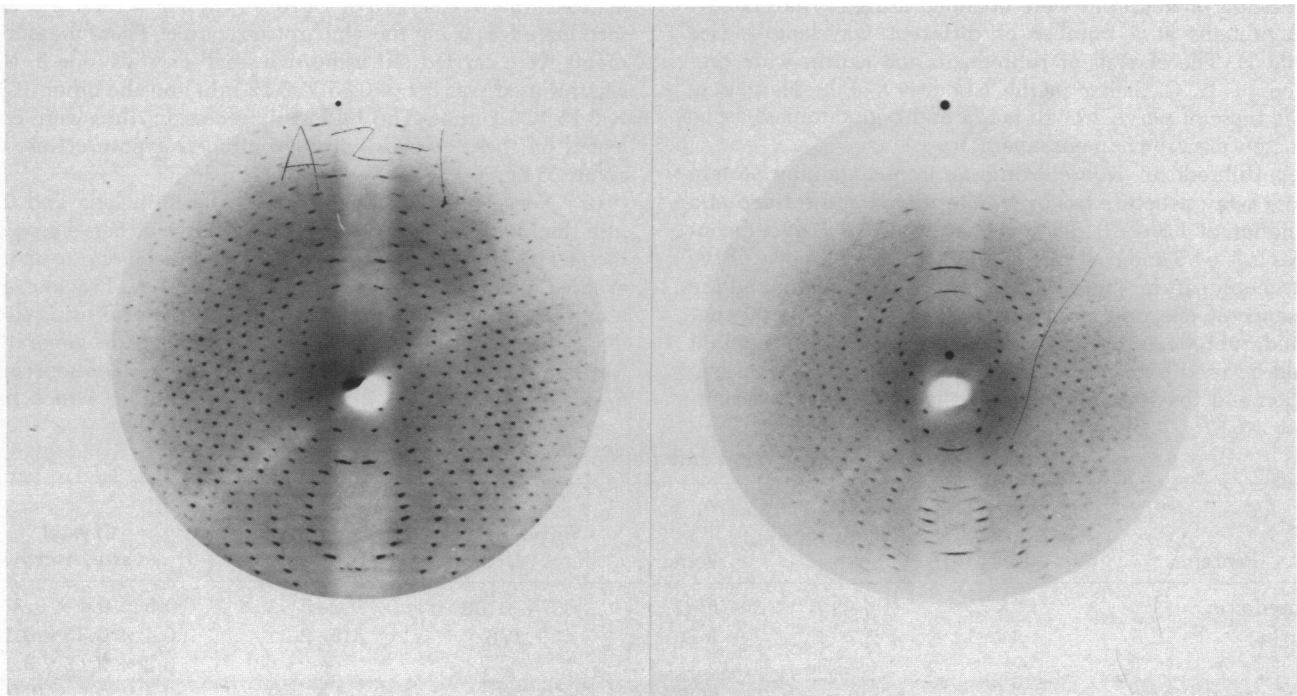

FIG. 3. Cone-axis oscillation photographs of the same azurin crystal. Precession angle 6.5°, oscillation angle 20°. (*left*) Synchrotron source, E = 3.7 GeV, I = 40 mA, only electrons present, λ = 1.740 Å, exposure time 10 min. (*right*) Philips fine-focus sealed Cu anode tube, operated at 40 kV, 30 mA, exposure time 6 hr, Ni filter.

permanent installation and higher operating energies and currents will result in even shorter exposure times for obtaining diffraction photographs.

The high intensity of the synchrotron radiation also enabled good quality precession photographs to be taken on crystals of nerve growth factor and L-glutaminase-asparaginase (see Table 1) which were smaller than normally considered adequate (13) and with which it was not possible to obtain useful photographs on similar crystals with our conventional sources.

A striking observation regarding protein crystal stability in this intense beam was made during the data collection of azurin. One of the two crystals used for the measurements accumulated a total of 28 hr exposure in the synchrotron beam. The crystal continued to diffract to at least 2.7 Å resolution after this irradiation. The radiation damage, as estimated from the time necessary to obtain precession photographs at the end of this period and from the unchanged diffraction resolution, was minimal. Similar behavior was observed with the other crystal. Intensity of diffraction from much larger crystals of azurin has been observed to decrease by about 25% after 100 hr of exposure with Ni-filtered radiation from a Cu tube operated at 700 W (L. C. Sieker, personal communication).

Thus, at least with these crystals, a time-dependent decay process is operative which is distinctly nonlinear in dose for x-rays, and even though decomposition could be proceeding at the same rate, data are being collected at least 60 times faster than with a sealed tube. This low rate of decay may in part result from the highly monochromatic nature of the x-ray beam, which has a bandpass of about 10^{-3} Å and is characterized by the complete absence of longer wavelength radiation which may be more damaging than the x-rays around 1.5 Å. A similar observation of increased lifetime of crystals (in this instance of a virus) was reported when radiation from a conventional tube was monochromated by a mirror-mirror focusing device (14). Because of the limited amount of data collected to date, these results are not quantitative. The other crystals which were used in these studies (nerve growth factor, glutaminase-asparaginase, and rubredoxin) also exhibited no abnormally rapid decomposition rates.

Tunability, Anomalous Scattering, and Collimation. Precession photographs were routinely measured at wavelengths ranging from 1.37 to 1.8 Å. At 3.7 GeV, there is substantial flux from the higher energy limit of about 0.5 Å (imposed by the critical energy of emission) down to the low energy limit of around 4 Å (imposed by the cutoff of the Be window). The usefulness of variable wavelength is principally in the ability to selectively maximize the real ($\Delta f'$) and imaginary ($\Delta f''$) components of the anomalous scattering factor for any element whose absorption edge occurs within the available wavelength range. Processing of the rubredoxin and azurin data has not yet been completed, but preliminary results on rubredoxin demonstrate significant changes when the magnitude of $\Delta f''$ is optimized by tuning to within 20 eV of the iron K absorption edge. Visual differences between Friedel pairs of reflections can be observed on the non-centric *hkO* plane. In several cases of relatively weak reflections, one of the Friedel pair will be absent while the other is clearly observed. As the wavelength is shifted away from the absorption edge, both reflections appear and become much closer in intensity. While details of numerical calculations using anomalous data will follow in a subsequent paper, preliminary data analysis is indicative of the anomalous scattering effects seen as a function of wavelength. For a 25° (*hkO*) precession film of rubredoxin taken on the low energy side of the Fe absorption edge ($\lambda = 1.780$ Å), R_{sym} for 110 Friedel pairs (whose background corrected intensities were obtained by scanning densitometry with $F_0 \geq 4\sigma$) was 2.0%, where R_{sym} is defined as:

$$R_{sym} = \frac{\Sigma 2(|F_{hkl}| - |F_{\bar{h}\bar{k}\bar{l}}|)}{\Sigma(|F_{hkl}| + |F_{\bar{h}\bar{k}\bar{l}}|)}$$

For a comparable film taken with Ni-filtered Cu Kα-radiation ($\lambda = 1.542$ Å), the R_{sym} was 2.6% for the same reflections. When taken at $\lambda = 1.740$ Å using the synchrotron source (just below the Fe K absorption edge where the imaginary component of the anomalous scattering ($\Delta f''$) should be maximized), the R_{sym} for several films ranged from 3.8 to 4.0%.

Radiation from the synchrotron source is also characterized by low angular divergence (crossfire). Estimates suggest that, after monochromatization by the Si crystal in the horizontal plane and collimation, the angular divergence of the beam should be a few milliradians. The divergence of the beam was in fact found to be so small that the size of the beam leaving the collimator was not measurably different from the beam recorded behind the camera, 30 cm away. The beam divergence is probably comparable to that obtained with a mirror-mirror focusing device (14). The resolution between reflections along the c^* axis on the nerve growth factor photograph (Fig. 2) was exceptionally good, considering that the length of the c axis is 181.4 Å and the film-to-crystal distance was only 6 cm. Mirror-mirror focusing would be required to obtain similar angular divergence characteristics from a conventional source (14).

Summary. The results from these preliminary studies clearly demonstrate that synchrotron radiation is a viable x-ray source for single crystal diffraction and that it offers several advantages over more conventional sources. The high flux of x-rays has allowed very rapid collection of intensity data on smaller protein crystals and to a higher resolution than would have otherwise been possible. This result on small crystals is quite important in view of the common difficulties in growing large enough single crystals of many proteins, even after conditions suitable for crystallization have been found. The stability of the crystals in the beam was greater than or comparable to their stability in a conventional source. The time necessary for alignment of protein crystals is dramatically reduced (e.g., 2 min for a 3° precession photograph), thus rapid survey and examination of large numbers of crystals and derivatives is facilitated. The highly monochromatic beam should result in improved signal-to-noise ratios because of the absence of background white radiation. While the enhancement of anomalous scattering has not yet been examined in detail, it is in principle possible to use data collected at three wavelengths (15) to completely solve the phase problem. The synchrotron source is uniquely suited for these applications.

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