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Anomalous Dispersion with Edges in the Soft X-ray Region: First Results of Diffraction from Single Crystals of Ribosomes Near the *K*-Absorption Edge of Phosphorus

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X-ray diffraction from a single crystal of the small ribosomal subunit of *Thermus thermophilus* was measured at five wavelengths near the *K*-absorption edge of phosphorus. The intensity of the low-order diffraction peaks varies strongly with the wavelength. It is influenced by the dispersion of contrast due to the P atoms of the ribosomal ribonucleic acid and by that of absorption, in comparable amounts.

Keywords: anomalous dispersion; ribosomes; absorption edges; soft X-ray diffraction; phosphorus *K*-edge.

1. Introduction

Since the advent of powerful synchrotron radiation sources, anomalous dispersion of X-ray scattering is increasingly used in molecular structure analysis (Hendrickson, 1991; Helliwell, 1992). It is intimately related to the excitation of inner-shell electrons, giving rise to an abrupt (anomalous) decrease of X-ray absorption and fluorescence with increasing wavelength. The X-ray absorption spectrum is characterized by a number of absorption edges named after the excited electron shells (*K*-, *L*-, or *M*-edges).

The wavelength range available from 1.2 to 7 Å at beamline A1 of HASYLAB covers the *K*-absorption edges of elements down to $Z = 14$ (silicon), including those of phosphorus ($Z = 15$) and sulfur ($Z = 16$). The latter elements are essential in living matter.

Phosphorus is a regular constituent of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). With three atoms in $1\,nm^3$, it is fairly abundant in nucleic acids. This also holds for the ribosomal RNA (rRNA), which represents two thirds of the total mass of the ribosome. Hence, the density of phosphorus might be regarded as sufficient for contrast variation studies, provided there is a means to change the scattering amplitude of the P atoms.

Phosphorus exhibits a strong dispersion of the real part (f') and the imaginary part (f'') of the atomic scattering amplitude (f) at wavelengths near the *K*-absorption edge (Fig. 1). Both f' and f'' vary by more than ten electrons in a narrow wavelength band $\Delta\lambda/\lambda = 2 \times 10^{-3}$ centred at $\lambda_K = 5.784$ Å, as demonstrated in this study. There is no similar behaviour of phosphorus at shorter X-ray wavelengths.

To exploit these effects one is confronted with serious absorption problems in the diffraction of soft X-rays. The penetration depth of 5.7 Å photons in air is 34 mm and in water it is reduced to 20 μm. Hence, the X-ray beam path has to be evacuated all the way from the source through the diffractometer to the detector. Clearly, the sample thickness should not exceed some tens of micrometers if forward scattering is to be observed. However, the situation is more convenient for backward scattering (Lehmann, Müller & Stuhrmann, 1993).

As the scattering is emerging from a very small sample volume, its intensity will be too weak unless synchrotron radiation is used. An instrument for diffraction experiments with soft X-rays has been installed at beamline A1 of HASYLAB at DESY, Hamburg (Stuhrmann, Goerigk & Munk, 1991). It has been used for preliminary X-ray diffraction studies from single crystals of lysozyme (Lehmann *et al.*, 1991), β-trypsin and the small subunit of ribosomes from *Thermus thermophilus* (Scholl *et al.*, 1994) at wavelengths near the *K*-absorption edge of sulfur. Here we report the first results of diffraction from a single crystal of the small subunit of ribosomes from *Thermus thermophilus* at wavelengths near the *K*-absorption edge of phosphorus.

2. Methods

The size of the crystals used in the experiments was around $30 \times 30 \times 100\ \mu m^3$. They were mounted on a copper needle with a flat top of 150 μm diameter. Controlled humidity and ambient pressure was ensured by an aluminium housing ($4\,cm^3$) with Mylar windows of 4 μm thickness, allowing for large scattering angles and rotations of the crystal. The copper needle was fixed to a cooling device consisting of a Peltier element connected to a heat exchanger.

Ribosome crystals are small, fragile and very sensitive to X-rays at room temperature. They are, therefore, kept at

temperatures below 100 K during exposure to X-rays and for storage (Hope *et al.*, 1989; Berkovitch-Yellin, Bennett & Yonath, 1992). As such, a low temperature could not yet be reached and maintained in the evacuated camera of our instrument. Hence, the ribosome crystal was cooled only to 268 K. Nevertheless, the crystals continued to diffract to very low resolution for 10 h at wavelengths near the *K*-absorption edge of sulfur and for about 4 h at wavelengths near the *K*-edge of phosphorus. The intensity of diffracted 5.0 Å photons is slightly higher than that obtained with 5.78 Å photons. We concentrate the discussion on the data obtained at the *K*-absorption edge of phosphorus, not only because it is the first measurement of anomalous diffraction from phosphorus in ribosome crystallography, but also because it is a striking example of contrast variation. The paper of Scholl *et al.* (1994) deals with data collection at 5.0 Å.

3. Results

Crystals of the small ribosomal subunit from *Thermus thermophilus* irradiated by 5.78 Å photons gave rise to at least ten low-order reflections near the beam stop at a fixed crystal orientation, which are well resolved by the multiwire proportional counter (Fig. 2). The reflections were indexed using the unit-cell dimensions $a = b = 407$, $c = 170$ Å, as determined by Yonath *et al.* (1988).

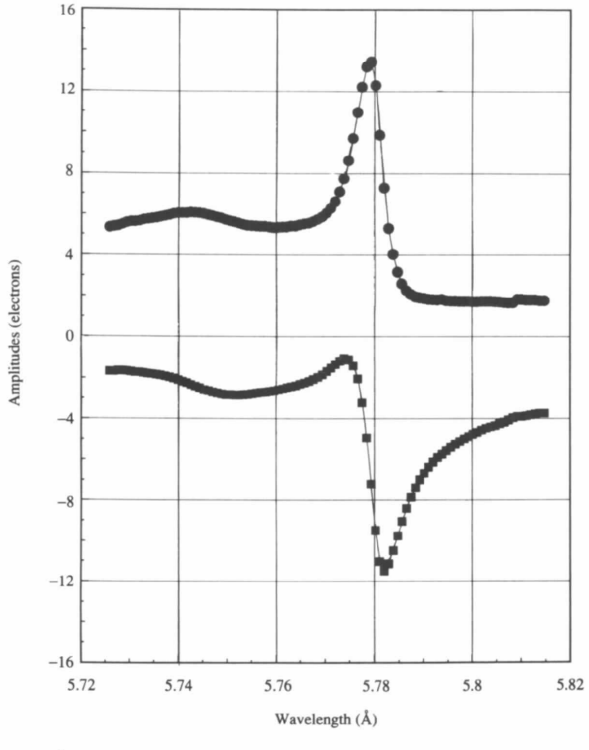

Figure 1
Anomalous dispersion of the scattering amplitude of phosphorus in ribosomal RNA. f'(■) and f''(●) vary by 11 electrons. The dispersion of f'' has been obtained from the X-ray absorption spectrum. The scale of f', as derived from f'' by using the Kramers–Krönig relation, has been derived from the anomalous dispersion of the X-ray small-angle scattering of a ribosome solution (Hütsch, 1993).

All reflections show a significant change in intensity when the wavelength is varied across the *K*-absorption edge of phosphorus, which could be readily followed on the screen during the experiment. A more detailed analysis shows that the variation of the scattering intensity is dominated by the dispersion of the absorption near the *K*-edge (Fig. 3). The diffracted intensity corrected for absorption is described by the following relation (Stuhrmann, 1985)

$$|F|^2 = |U|^2 + 2f'\mathrm{Re}[UV^*] + 2f''\mathrm{Im}[UV^*] + (f'^2 + f''^2)|V|^2, \quad (1)$$

where U is the amplitude of all atoms and $(f' + if'')V$ is the amplitude of the anomalous scatterers.

The analysis starts from data taken at three rotation angles of the crystals in steps of 0.2° and at five wavelengths, as shown in Fig. 3. The angular range was not large enough for the integration of low order peak intensities. This excludes the determination of the f''-dependent dispersion in (1) from the difference intensities of Bijvoet pairs. Hence, the variation of the intensity with the wavelength is analysed in terms of (1) for each diffraction spot, separately. As the spots occurring at very small diffraction angles (around 2°) show practically no change of their position with a relative variation of the wavelength of 0.005 Å, the analysis of the dispersion in the terms of (1) can be safely done with partial intensities.

4. Discussion

The number of counts per diffraction spot after subtraction of the background is a few hundred (per min). The background scattering near the beam stop varies strongly.

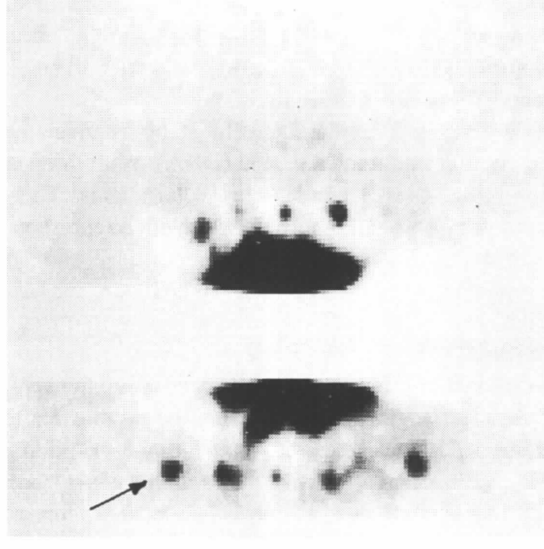

Figure 2
Diffraction of 5.8 Å photons from a single crystal of the small ribosomal subunit *Thermus thermophilus*. The background scattering has been removed except near the primary beam. The lower row of reflections from left to right can be indexed as (400), (310), (220), (130). The arrow points to the (400) reflection.

It is of the order of 1000 counts per peak area (*ca* $3 \times 3\,mm^2$). The statistical error of the spot intensities is around 15%. It limits the number of the basic terms in (1) to be determined from the experiment. Squared terms in (1) are neglected. As the dispersion of f'' is similar to that of the absorption, only the latter is considered. Hence, the variation of the diffraction spot intensities with the wavelengths shown in Fig. 3 will be discussed in terms of f' and the absorption. The functions $|U|^2$ and $[UV^*]$ and the f''-dependent absorption are determined by fitting the appropriately weighted dispersions of (1) to the intensities of a given Bragg peak measured at five wavelengths. As an example, the results from the (400) reflection are shown in Fig. 3. The variation of the real part of the amplitude is quite strong in all observed diffraction spots and comparable in size to the influence of absorption. The meaning of f' is known from small-angle scattering of solutions of ribosomes near the *K*-absorption edge of phosphorus: the contrast varies with f' (Hütsch, 1993).

This observation justifies the comparison of the crystallographic studies on ribosomes near the *K*-absorption edge of phosphorus with neutron diffraction from crystals of ribosomes (and viruses) in H_2O/D_2O mixtures (Eisenstein *et al.*, 1991). What matters is the change in contrast: it is given by the difference in scattering densities of the solvents, $6.96 \times 10^{10}\,cm^{-2}$. For phosphorus in rRNA, there are three P atoms per nm^3. The change in contrast due to the anomalous dispersion of phosphorus in rRNA is then $0.93 \times 10^{10}\,cm^{-2}$. Although this value falls far below that of solvent contrast variation in neutron scattering, the dispersion of X-ray scattering offers several advantages:

(*a*) The data are taken from the same sample, and hence systematic errors are smaller. In neutron scattering this could be achieved either by exchanging H_2O by D_2O in the same crystal or by nuclear spin contrast variation (Stuhrmann, 1993). The former method has hardly been used and the latter has not yet been tried for ribosome crystals.

(*b*) It provides the non-centrosymmetric term $\mathrm{Im}[UV^*]$, which is unavailable to contrast variation by isotope exchange. This is because the neutron scattering length of hydrogen has no imaginary part at thermal neutron energy.

(*c*) The meaning of the data is clear as there is no doubt that the phosphorus is an integral part of the ribonucleic acid. Methods of external contrast variation (*e.g.* neutron scattering in mixtures of H_2O/D_2O) reveal the spatial intraparticle scattering density fluctuations which in a further step can be associated with chemically different parts, like proteins and RNA in ribosomes.

(*d*) The scattering intensity can be increased by orders of magnitude at third-generation synchrotron radiation sources, provided freezing is used to control radiation damage to the sample.

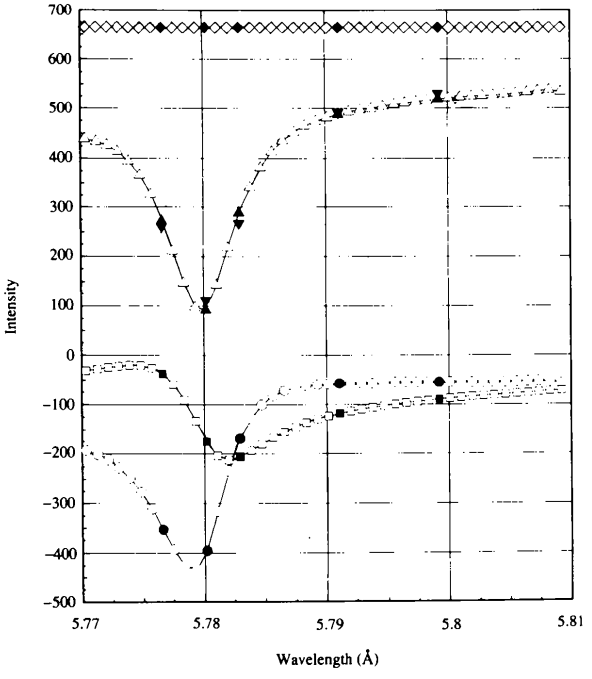

Figure 3
Dispersion analysis of the X-ray diffraction intensity for $hkl = 400$ at wavelengths near the *K*-absorption edge of phosphorus. Data were taken at wavelengths marked by full symbols. ▼ experimental, ▲ calculated dispersion using equation (1). Its components are: ◆ constant part, ■ f'-dependent intensity, ● variation of intensity due to absorption. The analysis is described in the text.

5. Conclusions

Diffraction of soft X-rays from crystals of large nucleoproteins, *e.g.* ribosomes, is feasible. To some extent this can be explained by the fact that the intensity of diffraction increases in a similar way to absorption. The ratio between the peak intensities of diffraction spots and background scattering does not change with wavelength.

The irradiated volume of the crystal does change with the wavelength. Since large crystals are not available, this is not a problem. However, the energy of the radiation is almost completely absorbed so that radiation damage is more serious and has to be minimized by freezing crystals.

It should be noted that the structural resolution is ultimately limited to 2.9 and 3.3 Å, in practice, using wavelengths of the *K*-absorption edges of sulfur and phosphorus, respectively, although much lower resolution has been used in these studies so far.

When the technical obstacles are fully overcome, anomalous dispersion of sulfur will allow phasing and hence structure determination of a wide class of proteins. Also, low-molecular-weight phosphates, *e.g.* ATP and GTP, may be used as labels. These have the advantage of a cumulated anomalous amplitude of more than 33 electrons.

The structure of RNA/DNA in viruses very often remains poorly resolved in crystallographic studies. Anomalous dispersion of phosphorus relates directly to this component

and therefore can help establish the structural relationship of the nucleic acid to the protein part of a virus.

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