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Electron radiation damage to protein crystals of bacteriorhodopsin at different temperatures

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Abstract

A series of diffraction patterns from two-dimensional protein crystals of bacteriorhodopsin (purple membrane) at different temperatures (294 K, 98 K and 4 K) were recorded as the diffraction spots faded due to radiation damage. The patterns were then computationally evaluated in order to obtain a quantitative measurement of the structural preservation while irradiating the specimen. To provide statistically significant results, diffraction spots corresponding to spacings of 3 Å (1200 spots) and 7 Å (600 spots) were measured. A substantial increase of the lifetime of high resolution spots was found using liquid nitrogen as a coolant, whereas further structural preservation at liquid helium temperature was significant but smaller. It appears likely therefore that high resolution images are accessible even at liquid nitrogen temperature. Mechanical stability and the absence of thermal specimen drift are certainly of equivalent importance for successful high resolution imaging.

1. Introduction

Radiation damage, the tiresome problem of electron microscopy, can in principle be overcome by sufficient cooling of the specimen (see, e.g., Refs. [1–6]). Therefore, by using cryo-electron microscopy at 4 K even the atomic structure of protein crystals could be solved [7,8]. However, the question remains open to what degree liquid helium temperature is necessary to preserve high resolution details. There are several reports on the effect of temperature dependent radiation damage, e.g. [9–15], but most results are either based only on visual fading of diffraction pattern or they do not provide *comparable* quantitative conditions for liquid nitrogen and liquid helium cooled specimen. From the general user's point of view, it would be very convenient if high resolution imaging can also be expected utilizing a conventional liquid nitrogen cooled device.

2. Methods

We have measured the dose dependent fading of electron diffraction patterns at 294 K (room temperature), 98 K (liquid nitrogen cooled) and 4 K (liquid helium temperature) using two-dimensional protein crystals of glucose embedded bacteriorhodopsin ("purple membrane"). For each measurement, six consecutive diffraction patterns of one individual crystal were recorded on a single micrograph, accumulating a dose of 0.5 e/Å^2 for each exposure on the specimen. This procedure has been developed by

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using remote control software for our microscopes [16].

Two different electron microscopes were employed for imaging: (i) a conventional Philips CM12 with a Gatan cryo-holder was used at 294 and 98 K and (ii) a special helium cooled Philips CM20 FEG with superconductive objective lens and field emitter illumination (SOPHIE) [17] for experiments at 4 K. We strictly attended to maintain identical conditions for each measurement and proceeded as follows:

(1) The *identical* specimen grid was used for all measurements.

(2) The illuminated area in the specimen plane and, thus, the dose accumulated on the specimen were adjusted to be measurably equal for each one of the microscopes. Beam current measurements were carried out in both cases by using the identical Faraday cup.

(3) For development of the photographic material standard conditions were maintained.

(4) Falsifications in the measurement due to specimen drift and thereby migration of unirridiated material contributing to the diffraction pattern [18] were excluded by choosing the diameter of the illuminated area to have twice the diameter of the selected area aperture.

More than fifty series of diffraction patterns were recorded for each temperature. Fifteen of the best series were selected in terms of their high resolution spots, digitised with a patch work densitometer [19] and stored in a computer for a quantitative evaluation. Those spots corresponding to spacings of 7 and 3 Å were extracted and the mean background noise was subtracted from the spot's intensity values. In this way a total of 1800 spots were evaluated, of which 1200 corresponded to spacings of 3 Å and 600 to those of 7 Å. To prevent saturation of the emulsion, the diffraction patterns were defocused, producing slightly broadened diffraction spots.

3. Results

Fig. 1 shows plots of the spot intensities for selected spacings (3 and 7 Å) and temperatures (294, 98 and 4 K) as a function of the radiation dose. The relative intensities of the reflections show in general an exponential fading with increasing electron dose. At room temperature only spots at 7 Å could be evaluated because of the fast decay of high resolution details. Comparing the decrease of the 7 Å spot intensities at different temperatures one recognizes a significant difference in the slope due to cooling from room temperature to 98 K. A "cryo-protection factor" C_p [20], which is given by the ratio of the critical dose N_e (defined as the dose at which the diffraction intensity has fallen to $1/e$ of its original value [10]) at different temperatures, has been determined to give a value of $C_p = N_e(98\ \text{K};7\ \text{Å})/N_e(294\ \text{K};7\ \text{Å}) = 9$. A further cooling to 4 K causes a further protection of high resolution details and the "lifetime" of 7 Å spots increases once more by the factor $C_p = N_e(4\ \text{K};7\ \text{Å})/N_e(98\ \text{K};7\ \text{Å}) = 1.4$ compared to liquid nitrogen temperature. Considering the smaller spacings of 3 Å, we obtain a cryo-protection factor of $C_p = N_e(4\ \text{K};3\ \text{Å})/N_e(98\ \text{K};3\ \text{Å}) = 2.5$ due to cooling from 98 K to liquid helium temperature.

4. Discussion

Commercially available microscopes are nowadays routinely operated within three major temperature ranges, namely room temperature, liquid nitrogen or liquid helium temperature. Suitable cold stages and transfer systems are available for each application. Unfortunately, investigations of the temperature-dependent cryo-protection of the specimen published earlier (see, e.g., Refs. [1–6], [9–15] and [21–23]), do not provide comparable results concerning the precise relative radiation damage. Different recording conditions, non-comparable samples or data analysis based on visual evaluation alone prevent a reliable assertion. The current investigation is the first of its kind, which provides *comparable* results on cryo-protection using the *identical* specimen at all those temperatures at which electron microscopes are normally operated. However, it should be mentioned that the exact specimen temperature during the illumination was not determined, but the large temperature gaps between 294, 98 and 4 K would be almost retained.

Our quantitative evaluations of electron beam induced decay rates confirm earlier works (Refs. [10] and [21–23]) reporting on an exponential decrease in spot intensities by increasing the electron dose. The

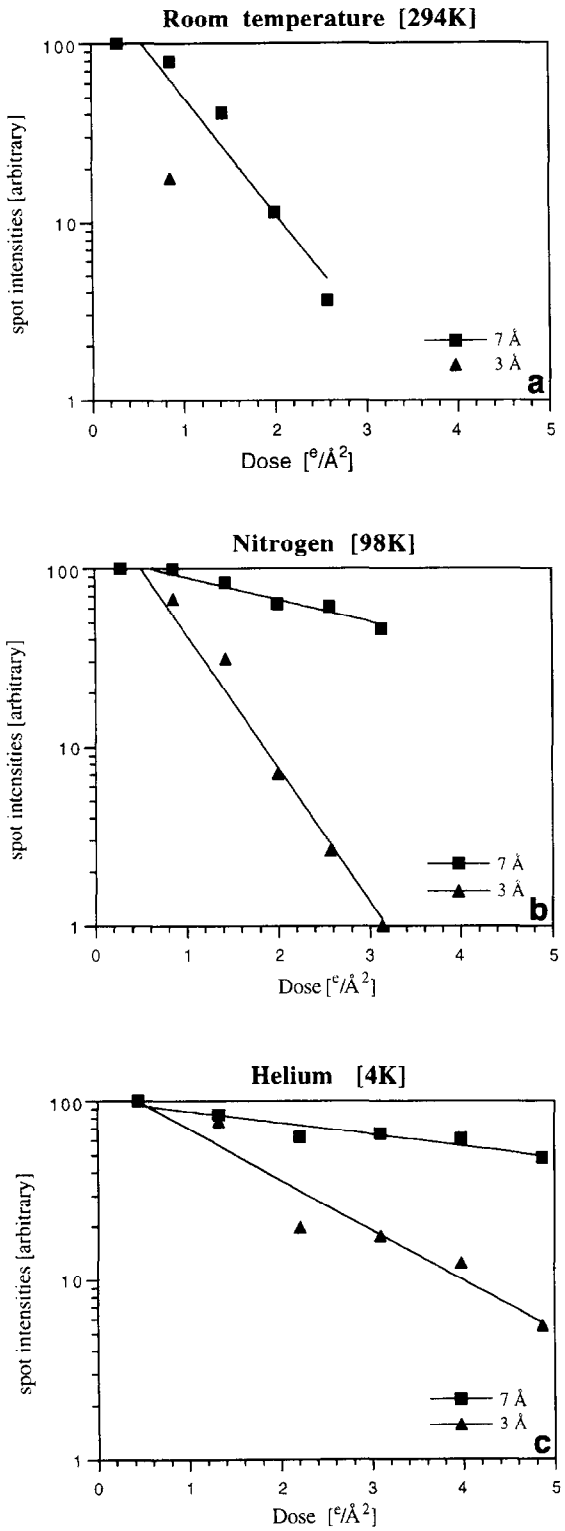

Fig. 1. The relative spot intensities of 3 Å and 7 Å spacings respectively as a function of the irradiation dose at 294 K (a), 98 K (b) and 4 K (c); (a) and (b) were measured with a conventional Philips CM12, (c) with a special helium-cooled Philips CM20 (SOPHIE) [17].

slope is temperature dependent. The most significant difference occurs by cooling the sample from room temperature to 98 K (the desired temperature of liquid nitrogen (77 K) cannot normally be achieved by using conventional cryo-holders because of insufficient thermal isolation). A cryo-protection factor of $C_p = N_e(98\ \text{K};7\ \text{Å})/N_e(294\ \text{K};7\ \text{Å}) = 9$ has been determined for reflections at 7 Å, which is approximately within the range determined by Hayward and Glaeser (1979) [10] from earlier experiments with purple membrane, who determined a protection factor of about 5 at 150 K. Surprisingly, the further cooling to the temperature of liquid helium did not reveal the dramatic increase of lifetime expected from the encouraging high resolution imaging at 4 K [7,24] and small cryo-protection values in the range $C_p = N_e(4\ \text{K};7\ \text{Å})/N_e(98\ \text{K};7\ \text{Å}) = 1.4$ to $C_p = N_e(4\ \text{K};3\ \text{Å})/N_e(98\ \text{K};3\ \text{Å}) = 2.5$ were found.

Considering the data over the full temperature range from 298 to 4 K (7 Å spacings), a protection factor of $C_p = N_e(4\ \text{K};7\ \text{Å})/N_e(298\ \text{K};7\ \text{Å}) = 13$ is obtained. A value of about 10 had been determined by Chiu et al. (1981) [12] for glucose embedded crotoxin complex crystals by cooling from 300 to 4 K. Later, the authors had discussed an even greater gain of about 50 in more detailed investigations [22], evaluating spacings between 2.2 and 20 Å. The differences in the results are most likely based on the differences in the specimen composition [25] and/or on effects of specimen drift as shown later [18]. They also depend on the spacings considered for evaluation [18]. Comparing the protection at 3 and 7 Å in our investigation the data reveal a slightly better structure preservation for the smaller 3 Å spacing values, $C_p = N_e(4\ \text{K};3\ \text{Å})/N_e(98\ \text{K};3\ \text{Å}) = 2.5$, than at 7 Å, $C_p = N_e(4\ \text{K};7\ \text{Å})/N_e(98\ \text{K};7\ \text{Å}) = 1.4$. It should, of course, be remembered that these results rather give a tendency and have to be considered with care due to the relatively large error bars, which are in the range of about $\pm 40\%$.

5. Conclusion

Our results indicate that cooling to liquid nitrogen temperature has the greatest impact on structure preservation. Mechanical stability of the specimen holder and the absence of specimen drift is hereby certainly of comparable importance for high resolution imaging. By using the helium cooled microscope SOPHIE [17] for high resolution imaging, specimen drift is, for example, significantly reduced and amounts to less than 0.05 Å/s. If these conditions were maintained in a liquid nitrogen cooled device, we would expect to obtain rather comparable results.

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Appendix A. Experimental section

A.1. Sample preparation

Two-dimensional crystals of bacteriorhodopsin were kindly provided by Dr. Richard Henderson (MRC). Droplets of the specimen were placed on freshly prepared hydrophobic carbon grids following the procedure described by Henderson et al. [7]. The excess fluid was blotted off with a filter paper and a second droplet of 0.8% (w/v) aqueous glucose solution was consecutively added for 1 min. The excess was again removed and the grid left to air dry. The same specimen grid was used for all measurments.

A.2. Image recording and dose measurement

The TVIPS CMONITOR microscope control software [16], which is routinely connected to our microscopes, allows the electron beam below the microscope objective lens to be shifted via a remote control "shift function" (Multi X, Y). This gave us the ability to install a procedure which consecutively records six diffraction patterns on a single micrograph. A "beam blank function" was introduced before and after each exposure time and during beam shift operations to avoid unwanted addititonal specimen irradiation. For an optimum use of the film area the diffraction camera lenghth was adjusted to be 770 mm at the recording plane.

In order to adjust the electron dose accumulated on the specimen during each exposure we proceeded as follows:

(i) A selected area aperture of 200 μm diameter, corresponding to an area of 4.562 μm^2 in the specimen plane, was used to limit the area contributing to the diffraction pattern. The entire illuminated area was then chosen to be twice the selected area diameter in order to avoid any falsifications due to specimen drift and gradual migration of the fresh unirradiated material into the diffraction area.

(ii) For a calibration of the microscope's screen voltage, which values were used for calculating the final dose, the beam current was measured directly by using a Faraday cup (Philips PW 6549/00). The electron dose of 0.5 $e/Å^2$ which was accumulated for each exposure was then adjusted by using the microscope condenser lens (K2). Screen voltage measurements were proved to be equal before and after each series recordings.

The patterns recorded on Agfa Scientia EM Film 23D56 were immediately developed at 20°C for 12 min by using Kodak D19 (full strength) developer.

A.3. Image analysis

The micrographs were digitised by using a "patchwork" densitometer [19] with a sampling grid corresponding to 20 μm. All image analysis and densitometry were performed in the context of the IMAGIC V software system [26] on DIGITAL 3200 workstations. Individual diffraction spots of 3 and 7 Å respectively were extracted. The intensity of the non-specific scattering background was averaged and subtracted from the measured diffraction spot intensities.

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