

# DEVELOPMENT OF AN OPEN-FLOW CRYOSTAT UTILIZING HELIUM GAS FOR CRYOGENIC X-RAY DIFFRACTION EXPERIMENTS

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An open-flow cryostat utilizing helium gas as a cryogen has been developed for cryogenic X-ray crystallography. The device composed of a cryostat and a heat exchanger is easy to operate. Nitrogen gas is supplied to shield the cooled helium gas. The lowest temperature reached by the device is 35 K within a temperature fluctuation of 0.5 K at a flow rate of 6 Liter/ min. This device facilitates structural studies between 35 K and 90 K.

## 1. Introduction

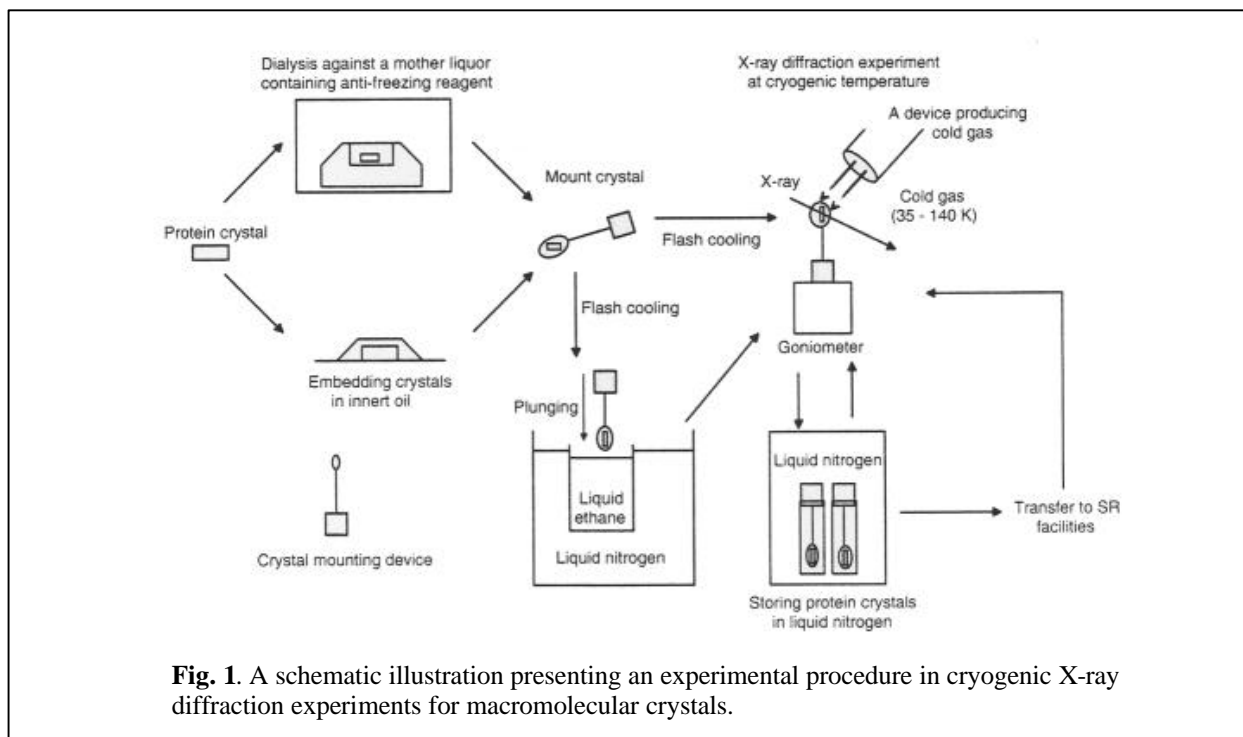
Protein crystallography has made remarkable progress in the last decade under the developments of area detectors with Imaging Plate or charge-coupled devices, third-generation synchrotron X-ray sources, cryogenic X-ray diffraction techniques and software for data processing and structure analyses [1]. Now, protein crystallography has been successfully applied to determine huge protein complexes [2-4] and is regarded as the most important tool for carrying out many structural genomics projects targeting a numerous number of biological macromolecules in several organisms.

Cryogenic X-ray diffraction techniques [5] reducing the radiation damage of biological macromolecular crystals have provide reliable diffraction data and enable to store crystals in liquid nitrogen baths for long periods. Radiation damage of protein crystals is caused by radicals and Auger electrons produced when crystals absorb X-ray photons. In particular, radicals from water molecules occupying a large volume of the crystals probably destroy the intermolecular interactions at the crystal contact area in the diffusion processes. Therefore, to overcome the radiation damage of macromolecular crystals, it is the easiest and best way to freeze the diffusive motions of the radicals under cryogenic temperatures below the glass transition point of water.

A cryogenic technique was first applied to the diffraction data collection of insulin crystals in 1966 [6]. Since then, many technical innovations

and improvements have been proposed and attempted [7-17], and now cryogenic technique is indispensable to collect reliable diffraction data of high quality in synchrotron facilities. In addition, the technique has great potential to deal with very thin or fragile macromolecular crystals, which can not be treated softly in capillary tubes. In Japan, the technique has been developed by one of the authors since 1994 [18-21] toward effective use of undulator X-rays at SPring-8, a third-generation synchrotron facility. Figure 1 illustrates schematically the procedures in cryogenic experiments performed in our laboratory. The technique has now been used for studying the hydration structures [22-31] and the vibrational states of proteins under cryogenic temperatures [32-34]. However, some problems remain to be examined and challenged: extension of the temperature range [35, 36], methods in researching systematically anti-freezing reagents suitable for crystals, the control of the cooling rate, and the cooling procedure for mm-size crystals towards cryogenic neutron crystallography.

Until now, with a few exceptions, cryogenic diffraction data collections have been usually performed around 100 K, which is below the glass transition temperature of water. At this temperature, radiation damage in macromolecular crystals was little in experiments [37] at laboratories and some beam-lines at the Photon Factory, a second-generation synchrotron facility. In contrast, when ultra-thin protein crystals are exposed for a long period to X-rays from a bending magnet of



SPring-8 (ex. BL44B2 [38]), the degradation of the crystals even at 100 K is clearly seen as decreasing the effective resolution limits and increasing the apparent temperature factors of the crystals (Nakasako, unpublished results). In addition, many researchers reported radiation damage even at 100 K in the diffraction experiments [39] performed at BL41XU [40] of SPring-8, where intense undulator X-rays were available. These facts suggest that a numerous number of X-ray photons may cause melting of vitreous ice in crystals repeatedly, and that radicals can diffuse in local areas in a short period. As shown in electron microscopic observation of biological samples [41], measurements at lower temperatures result less radiation damage in the samples. Therefore, we started to develop a cooling device facilitating diffraction experiments below 90 K. Here, we describe the development of a cryocooling device enabling cryogenic diffraction experiments at 35 K and the preliminary X-ray diffraction experiments using this device at BL41XU of SPring-8.

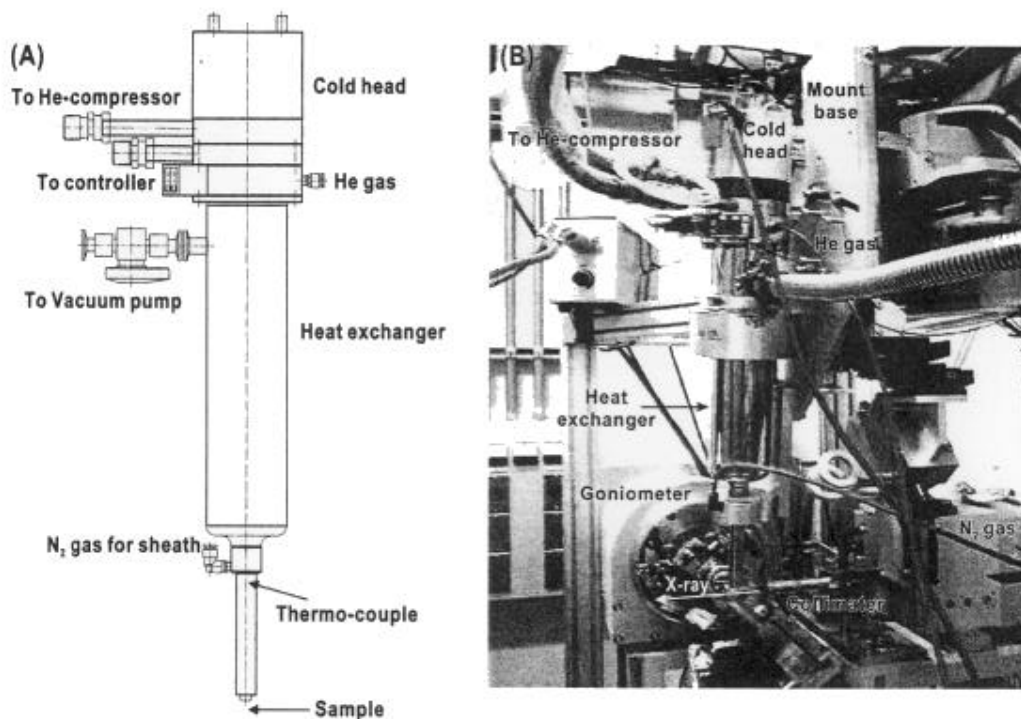
## 2. Design Concept and Specification of the Open-Flow Cooling Device Developed

When we started developing the new cooling device, a cryo-cooling device utilizing nitrogen gas as a cryogen had been developed by Rigaku Corporation. In that device, nitrogen gas is cooled

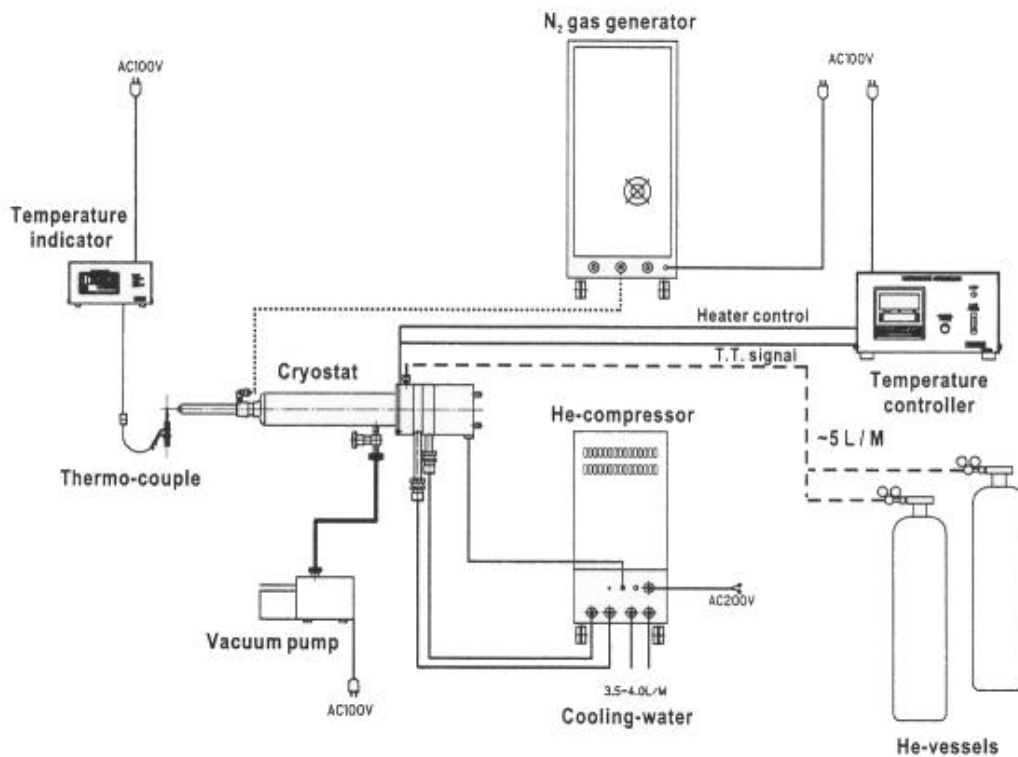
by a heat exchanger attached to the cold head of a cryostat. This method is quite simple and easy to operate. In designing the new device, we also adopted this method, but changed the cryogen from nitrogen gas to helium gas, the boiling point of which is 4 K.

Figures 2 and 3 schematically show the overview of the newly developed device. As a refrigerator, we selected a D510 cryostat by IWATANI having a two-stage cold head and working under a modified Sorvey cycle. The temperature of the first stage reaches to 38 K, and that of the second stage does to 10 K. Because the length of the cold head is twice that used in the cryo-device utilizing nitrogen gas, the heat exchanger is also double-sized. The large volume of the heat exchanger ensures effective heat transfer between the cold-head and helium gas. The heat exchanger and the cold head are isolated thermally from the atmosphere by a stainless Dewar. In the exit nozzle attached to the end of the Dewar, a ceramic heater is set inside to control the temperature of cooled helium gas. The temperature is monitored at the end of the exit nozzle by a thermocouple, and a controller regulates the load of the heater through monitoring the thermoelectromotive force of the thermocouple.

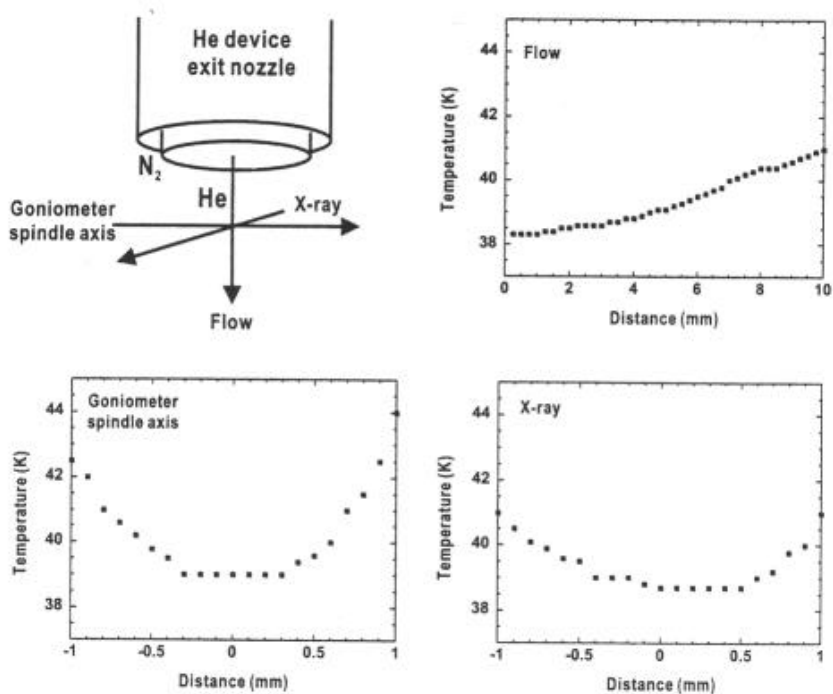
After constructing the prototype device, we



**Fig. 2.** A draft (A) and a photograph (B) of the He-cooling device. The photograph was taken at BL41XU of Spring-8 after setting the device on the goniometer carriage.



**Fig.3.** A schematic illustration of the He-cooling system. The dashed and dotted lines indicate the supply of helium gas and dry nitrogen gas, respectively.



**Fig. 4.** Temperature variation around the exit nozzle of the He-cooling device measured with a thermocouple at a He-gas flow rate of 5.0 Liter/min.

examined which of nitrogen or helium gas shields effectively the cooled helium gas from the atmosphere. When helium gas was supplied as the 'sheath' for the cooled gas stream, the temperature of the cooled helium gas fluctuated widely. In contrast, when nitrogen gas was supplied, the temperature of the cooled helium gas was stabilized nicely. The viscosity of nitrogen gas is advantageous to form a cylindrical sheath guiding and restraining the flow of the cooled helium gas. The specific heat of nitrogen gas is also efficient in reducing heat conduction between cooled helium gas and the sheath. As a result, we choose nitrogen gas, and the gas is supplied from a nitrogen gas generator at a flow rate of 5.5 Liter/min.

When helium gas was supplied to the cold head at a flow rate of 6.0 Liter/min, the temperature of the cooled helium gas reached 35 K within 1 hour after starting the cold head. The stability of the temperature at the sample position was less than 0.5 K at 35 K. Then, inside the exit nozzle, the temperature of the cooled helium gas was 22 K. At the edge of the exit nozzle, temperature of the cooled gas was raised by 10 K because of the

heat conduction to the edge. A modification to the edge region may improve the heat conduction. Figure 4 shows the temperature variation of the cooled helium gas near the nozzle of the device. There is a plateau of 1 mm along the horizontal direction and the temperature is stable along the flow within 10 mm from the tip of the nozzle.

In a test run of the device, it was found that the device should be stood within 10 degrees from the normal direction. This is probably due to the fluid dynamic properties of helium gas. When the device is inclined more than 30 degrees from the normal, the temperature at the exit nozzle was more than 50 K and fluctuated widely. In addition, when the device is directly mounted on a base plate attached to a goniometer, the vibration caused by the Sorvey-cycle in the cold head transmits to the goniometer. To avoid this, three silicon blocks of ca. 20x20x20 mm<sup>3</sup> are used for insulating mechanically the device and the mounting arm, resulting in a complete damping of the vibration.

Beamline	BL44B2	BL41XU
Crystal size (mm <sup>3</sup> )	500 x 200 x 50	500 x 200 x 50
Temperature (K)	110	37
Space group	P21 (60,000 (trimer)/asymmetric unit)	
X-ray wavelength (Å)	0.700	0.710
Oscillation (deg/sec)	0.75/20	0.6/2
Total diffraction patterns	300	300
Lattice constant a (Å)	72.63	72.64
b (Å)	61.29	61.30
c (Å)	72.62	72.62
β (°)	120.02	120.02
Resolution (Å)	100.0 – 1.65	100.0 – 1.45
Number of reflections	261,284	365,877
Unique reflections	69,912	98,206
Redundancy	3.7	3.7
Completeness (%) (last shell)	99.9 (100) <sup>a</sup>	99.7 (100) <sup>b</sup>
I/σ(I) (last shell)	30.7 (3.4) <sup>a</sup>	23.8 (4.2) <sup>b</sup>
R <sup>I</sup> <sub>merge</sub> (I>1σ(I)) <sup>c</sup>	0.037 (0.300) <sup>a</sup>	0.043 (0.297) <sup>b</sup>

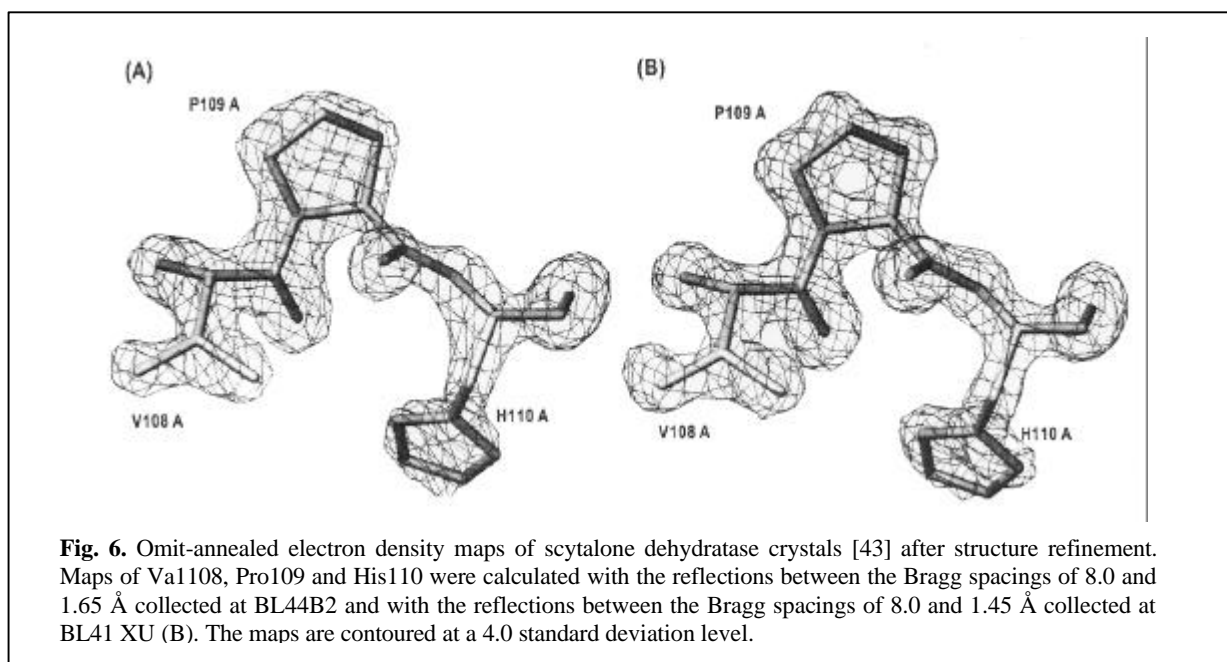
<sup>a</sup> Last shell 1.47 to 1.45 Å <sup>b</sup> Last shell 1.47 to 1.45 Å <sup>c</sup>  $R_{merge}^I = \frac{\sum_h \sum_i |I_i(h) - \langle I(h) \rangle|}{\sum_h \sum_i I_i(h)}$ , where  $I_i(h)$  is the intensity of the I-th observation of reflection.

### 3. X-Ray Diffraction Experiments Using the Device at BL41XU of SPring-8 [42]

Here, we describe preliminary experimental results using the device at BL41XU of SPring-8. Over a beam time of 4 days, the device worked well, and no trouble occurred in the operation. During the experiments, we collected diffraction data from crystals of various proteins and macromolecular complexes using a MarCCD165 detector. As an example, Table 1 shows the statistics of

the diffraction data collected from a thin-plate crystal of scytalone dehydratase [43].

The collected data is compared with that collected at BL44B2 of SPring-8 in Table 1 and Fig. 5. In both the experiments, there are no signals indicating degradation of the crystals. In particular, owing to the good spatial coherence of the X-ray beam available at BL41XU, the resolution limit of the data was markedly extended to a resolution of 1.45 Å in the present experiment (Table 1, Figs. 5 and 6).



Crystals of biological macromolecules are usually cooled and stored in a liquid nitrogen bath. In cryogenic X-ray diffraction experiments at synchrotron facilities, crystals stored in a liquid nitrogen bath are directly mounted on a goniometer. Then, the liquid nitrogen adhering on cooled crystals is transformed to solid nitrogen, when crystals mounted at 35 K. Strong Debye-Scherrer rings from solid nitrogen appearing in the diffraction pattern make it difficult to evaluate the diffraction intensities from protein crystals around the rings. To avoid this, the mounting of crystals should be carried out near 90 K. After liquid nitrogen vapors, the temperature of cooled helium gas is set at 35 K.

In many synchrotron beam-lines, the goniometer is set in horizontal geometry. Therefore, stainless pins of the commercially available crystal-mounting device (Hampton Research, USA) run across the sheath gas region. In some cases, a thin solid-nitrogen layer is formed on the mounting-device from the sheath to the sample regions, because of the turbulent flow around the pin. To avoid this, we usually glued a small drop of an epoxy resin or a small circular plate at the tip of the pin to deflect the nitrogen gas prior to cooling crystals.

### Conclusion

We successfully developed an open-flow type cryo-cooling device utilizing helium gas as a

cryogen. The device is easy to operate and stably runs for long periods. The device facilitates diffraction experiments for macromolecular crystals at 35 K and may be utilized to investigate temperature-dependent phenomena between 90 K and 35 K.

### Acknowledgement

The authors thank Dr. S. Togitani of IWATANI Corp., Mr. H. Yokozawa of Rigaku Corp., Mr. Y. Yamada of Union Engineering Corp. and Prof. N. Kamiya of RIKEN Harima for their fruitful discussions and help in developing the cooling device. This study was supported by a grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. The X-ray diffraction experiment at BL41XU of SPring-8 was carried out with the approval of the organizing committee of SPring-8 (proposal No. 2000B0097-NL-np).

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