J. Appl. Cryst. (1997). **30**, 190–194

Successful flash-cooling of xenon-derivatized myoglobin crystals

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(*Received 18 July 1996; accepted 16 October 1996*)

Abstract

This paper demonstrates for the first time a method for preparing cryocooled xenon-derivatized protein crystals. The method is based upon the hypothesis and subsequent observation that the diffusion of a xenon atom from a tight binding site following depressurization occurs on a timescale of minutes. We have observed significant changes in diffraction intensities from myoglobin crystals for up to 5 min following depressurization from 1 MPa of xenon. In accordance with this observation, a xenon-derivatized myoglobin crystal was cryocooled at ~95 K within 20 s of complete depressurization. A crystallographic data set was then collected to 2.0 Å resolution and isomorphous and anomalous difference Patterson maps revealed the presence of a well ordered xenon site with an occupancy of approximately 0.5. Phasing statistics for this site were of good quality and demonstrate the practicality of this method. The ability to cryocool xenon-derivatized crystals will make this heavy-atom substitution method even more useful for single-isomorphous-replacement and multiple-isomorphous-replacement phasing of macromolecules.

1. Introduction

It is now well established that xenon binds to numerous proteins (see Table 1). We have observed an approximate 50% success rate where useful phasing statistics have been obtained (Stowell *et al.*, 1996). Similar statistics were recently reported by Prange, Schiltz & Fourme (1996). There are a number of advantages to the use of xenon as a heavy-atom derivative: the derivative crystals can be extremely isomorphic with the native crystals, xenon binds to sites that are unique to most conventional heavy-atom sites and the number of binding sites can sometimes be controlled simply by changing of the pressure.

The preparation of xenon derivatives or the incubation of macromolecular crystals with gases to study ligand binding has prompted the development of suitable cells for such purposes (Stowell *et al.*, 1996; Schoenborn, Watson & Kendrew, 1965; Tilton, Kuntz & Petsko, 1984; Tilton, 1988; Kroeger & Kundrot, 1994; Shiltz, Prange & Fourme, 1994). There are disadvantages to the use of a pressure cell for X-ray diffraction experiments, however. A major disadvantage is the inability to cryocool the sample (Hope, 1988), which has been shown to help prevent radiation damage due to the intense X-ray beam. Another disadvantage of a pressure cell is the increase in background in the X-ray diffraction pattern as well as the attenuation of the X-ray beam by the pressurized gas. In addition, whenever glass or quartz capillary tubes are pressurized, there exists an explosion hazard. Accordingly, it is desirable to develop methods that will allow the pressurization and cryocooling of gas-pressurized samples. Cryocooling affords a significant reduction in radiation damage to the crystal, less background in the X-ray diffraction pattern and provides a simple mounting technique for samples. In addition, the formation of xenon hydrates may be avoided. Herein, we describe experiments to demonstrate the feasibility of preparing cryocooled xenon-derivatized protein crystals. We chose to begin our studies with myoglobin since it has a well characterized xenon binding site (Schoenborn *et al.*, 1965; Tilton *et al.*, 1984; Schoenborn, 1969; Vitali, Robbins, Almo & Tilton, 1991). We monitored the intensity changes in the diffraction pattern from a myoglobin crystal during pressurization and depressurization with xenon gas at room temperature. The results indicate that xenon diffuses from the crystal after

Table 1. *Protein crystal derivatization using xenon gas*

Reproduced in part from Stowell *et al.* (1996). BPI human bactericidal permeability-increasing protein; CAM carbonic anhydrase, *Methanosarcina thermophila*; CHIP Human erythrocyte aquaporin; C554 cytochrome c554, *Nitrosomonas europaea*; DMSOR dimethyl sulfoxide reductase, *Rhodobacter spheroides*; HHB horse hemoglobin; HLC collagenase, *Hypoderma lineatum*; IMPDH inosine monophosphate dehydrogenase, *Tritrichomonas foetus*; ORF2 nitrogen fixation specific open reading frame 2, *Azotabacter vinelandii*; PPE porcine pancreatic elastase; RXR-a human retinoid-X receptor alpha; STCN stelacyanin, cucumber; MB sperm whale myoglobin; MBA alkaline sperm whale myoglobin; SC subtilisin Carlsberg, *Bacillus licheniformis*; SOD Superoxide dismutase, *Saccharomyces cerivisiea*.

Protein	MW (KDa)	Xenon pressure (MPa)	Patterson	Difference Fourier	Number of sites	Unique sites
BPI	50	1		Yes	4	Yes
CAM	23	0.4–1	Noniso			
CHIP	28	0.4–1	No			
C554	25	1	Poor		3	
DMSOR	85	1	Yes		1	Yes
HHB	67	0.3			1	
HLC	25	1.2			1	Yes
IMPDH	58	0.4–1	Noniso			
LYS	14	0.8	Poor		4	
MBA	18	0.3		Yes	2	
MB	18	0.3		Yes	1	
MB	18	0.7		Yes	4	
MB	18	0.7	Yes		4	
ORF2	30	1.2	Yes	Yes	1	No
PPE	26	0.8	Yes		1	
RXR-a	30	2	Yes		2	Yes
SC	27	1.2		Yes	1	
SOD	16	1		Yes	1	Yes
STCN	12	1		Yes	1	Yes

5 min following depressurization to atmospheric pressure. These results prompted us to cryocool a sample immediately following depressurization, with hopes of trapping xenon in the binding site.

2. Xenon binding/release studies

X-ray diffraction data were recorded from a hexagonal (*P*6) crystal of sperm-whale myoglobin mutant (Asp122 to Asn) (Phillips, Arduini, Springer & Sligar, 1990) mounted in a pressure cell (Stowell *et al.*, 1996) using a MAR Imaging Plate Scanner System on beamline 7-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). The same 1° rotation was recorded for each image. The X-ray exposure time was 10 s (time resolution) and the time for detector read-out, erasure and φ-axis reset was approximately 110 s (sampling rate). Data were collected before, during and after pressurization with 1 MPa of xenon gas at room temperature. Data reduction was carried out using the *HKL* package (Otwinowski & Minor, 1993) and 2282 intensities for fully recorded reflections were output unmerged using *SCALEPACK*. Fig. 1(*a*) shows the change in intensity for six reflections. The times at which the cell was pressurized and vented are indicated in the figure. After the initial change in intensity following the pressurization step, we observed gradual changes of relatively small magnitude over a period of ~1 h. In addition, following the depressurization step, the rate of change of the individual intensities varied. These observations may be a result of the differing binding rates for three additional xenon binding sites in myoglobin (Tilton *et al.*, 1984; Vitali *et al.*, 1991), although these sites have lower occupancy. Also, there were reflections that did not return to their original intensity after depressurization. This may be an indication of residual xenon or that partial dehydration has occurred. The changes in intensity of 60 reflections for which the intensity increased after pressurization have been averaged and are plotted in Fig. 1(*b*) and expanded in Fig. 1(*c*). We made no attempt to measure the

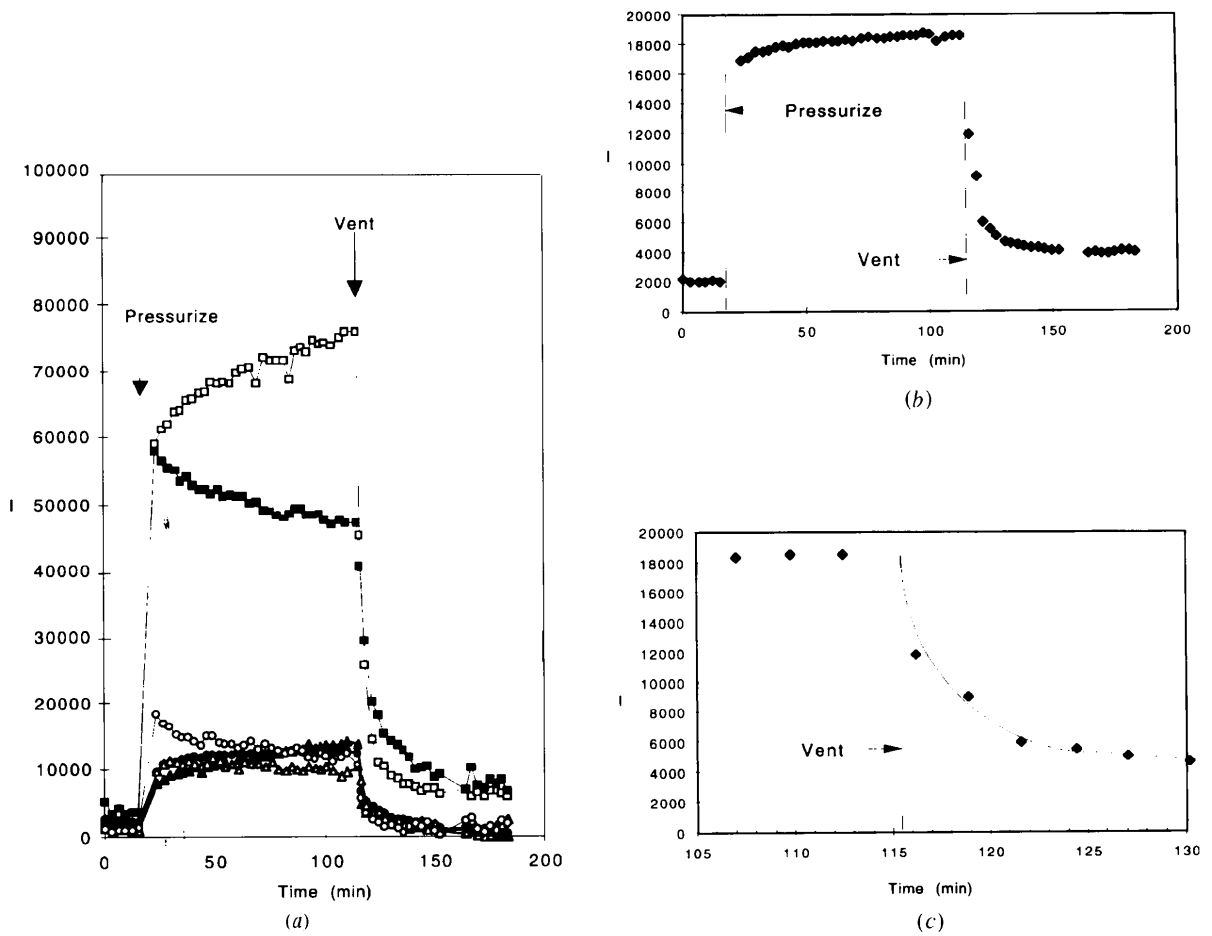


Fig. 1. (*a*) Intensity changes as a function of time for six individual reflections. The times at which the cell was pressurized and vented are indicated. (*b*) Average intensity for 60 reflections that increased in intensity during xenon pressurization. (*c*) Enlarged view of the xenon depressurization step in (*b*).

Table 2. *Data-collection statistics for cryocooled myoglobin*

	Native	Xenon derivative
Unit cell		
a (Å)	89.94	90.19
b (Å)	89.94	90.19
c (Å)	45.14	45.22
Resolution (Å)	15–2.0	15–2.0
Completeness (%)	93	96
Multiplicity	3.1	3.2
R_{sym} (%)	7.1	3.2
R_{iso} (%)	16.2	

initial binding time, but we can say that xenon binding is nearly complete after 10 min (see Fig. 1*c*) in accordance with a previous report (Schiltz *et al.*, 1994). Xenon diffusion from the crystal is nearly complete after 5 min.

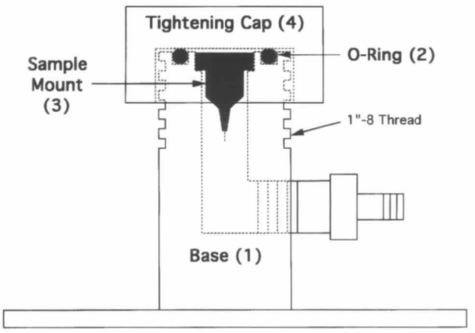
3. Pressure/cryo device

Fig. 2(*a*) shows a schematic of a prototype pressure cell for quick cryocooling of samples after depressurization. Fig. 2(*b*) shows an exploded view of the pressure cell. The steel base (1) is composed of a hollow shaft that houses the sample; a standard stainless steel Swagelok fitting is used for connection of a gas-delivery tube. The sample is mounted on a loop that is fixed to the mounting pin with cement. The pressure cell uses an *O*-ring seal (2) against the bottom of the cap (4). A magnetic steel mounting pin (3) rests in the base. Manipulation of the mounting pin is performed with magnetic hemostats (5).

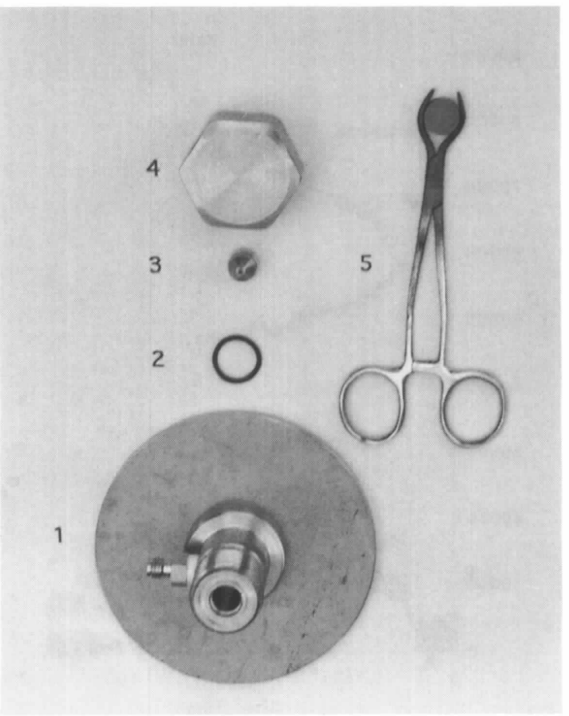
The pressure cell is simple and safe to use. A piece of filter paper saturated with mother liquor, or a small vial containing mother liquor, can be placed in the cell to prevent crystal dehydration. The crystal is mounted on a loop affixed to the steel mounting pin using standard cryocrystallographic mounting techniques. The mounting pin is inserted into the base using magnetic hemostats. The long thread on the base prevents the cap from being propelled should the cell be opened while it is pressurized. When the cell is depressurized, the cap can be swiftly removed, allowing the pin to be quickly transferred directly into liquid nitrogen, thereby flash-cooling the sample. The shortest time between depressurization and cryocooling that can be achieved using this device is approximately 7 s. Once the sample has been cooled in liquid nitrogen, it can be transferred to an X-ray camera equipped with a nitrogen-gas cold stream using cryocrystallographic transfer tongs (Hope, 1995).

4. Cryocooling of xenon-derivatized myoglobin

A myoglobin crystal mounted on a nylon loop was dipped in succession into three fresh drops of cryoprotectant solution for ~2 s in each. The cryoprotectant solution was made by the addition of 250 mg sucrose to 1 ml of 70%-saturated ammonium sulfate with 0.050 *M* Tris-HCL, pH 9.0. A drop of cryoprotectant solution was added to a pressure cell to prevent the crystal and loop from drying out and the sample was pressurized with 1 MPa of xenon gas for 10 min at room temperature. The pressure was then released and the myoglobin crystal was cooled to a temperature of ~95 K directly in a cold nitrogen gas stream (Bellamy, Phizackerley, Soltis & Hope, 1994) on beamline 7-1 at SSRL. The time over which the pressure was released was about 10 s and the time between complete depressurization and cryocooling was approximately 20 s. A complete data set to 2.0 Å resolution was collected in just under 1.5 h and was processed using the *HKL* package (Otwinowski & Minor, 1996). In addition, a native data set was collected at a cryogenic temperature. Data-collection statistics for both data sets can be found in Table 2. Scaling statistics gave no indication that xenon was escaping from the crystal



(*a*)



(*b*)

Fig. 2. (*a*) Schematic and (*b*) exploded view of a pressure cell for quick cryocooling of protein samples. 1 Steel base; 2 *O*-ring seal; 3 magnetic steel mounting pin; 4 cap; 5 magnetic hemostats.

Table 3. *Single-isomorphous-replacement analysis for cryocooled xenon-derivatized myoglobin*

Phasing power = $\langle|FH|\rangle/\langle|\varepsilon|\rangle$, where $\langle|FH|\rangle$ is the root mean square of FH and ε is the root mean square of the lack of closure. Cullis $R = \sum|FH(\text{obs.}) - FH(\text{calc.})|/\sum|FH(\text{obs.})|$.

	Acentric reflections				Centric reflections				
Resolution shell (Å)	Number	Figure of merit	Phasing power	Cullis R	Number	Figure of merit	Phasing power	Cullis R	Figure of merit (total)
9.02	89	0.33	1.65	0.80	27	0.61	1.33	0.60	0.40
6.02	312	0.39	1.81	0.68	53	0.68	1.43	0.64	0.44
4.52	633	0.37	1.45	0.72	78	0.63	1.17	0.75	0.40
3.62	1047	0.28	1.02	0.83	91	0.50	0.97	0.75	0.29
3.02	1599	0.30	1.05	0.82	113	0.55	0.89	0.73	0.32
2.59	2228	0.31	1.17	0.79	136	0.57	0.99	0.69	0.33
2.27	2935	0.30	1.16	0.82	125	0.53	0.96	0.79	0.31
2.01	3541	0.29	1.09	0.83	135	0.51	0.85	0.77	0.29
Total	12384	0.30	1.15	0.81	758	0.56	1.01	0.73	0.32

during data collection, as would be expected from the relatively high melting point of xenon, 137 K, compared to the temperature of data collection, ~95 K.

Data analysis was carried out with the *CCP*4 package (Collaborative Computing Project, Number 4, 1994). The xenon site was located by difference Patterson analysis. The strongest peak in the Harker section of an isomorphous difference Patterson synthesis computed with data in the resolution range 10 to 2.0 Å was 16 times the standard deviation of the map (Fig. 3). The position and occupancy of the Xe atom were refined using *VECREF* and phases were calculated using the program *MLPHARE* (Table 3). Our cryocooled xenon derivative proved to be of good quality with an overall figure of merit of 0.32 to 2.0 Å. It should be noted that Vitali *et al.* (1991) have demonstrated that phases from a single Xe atom in myoglobin are sufficient to produce an interpretable electron-density map for the structure of myoglobin.

We have observed that xenon binds in less than 10 min in the case of myoglobin. This observation is similar to that for porcine pancreatic elastase (Schiltz *et al.*, 1994). We concur with those authors that xenon binding appears to take place on a timescale of minutes rather than hours. From the calculated occupancy of the xenon site in myoglobin, we can estimate the half-life for the xenon off-rate to be of the order of 20 s. Thus,

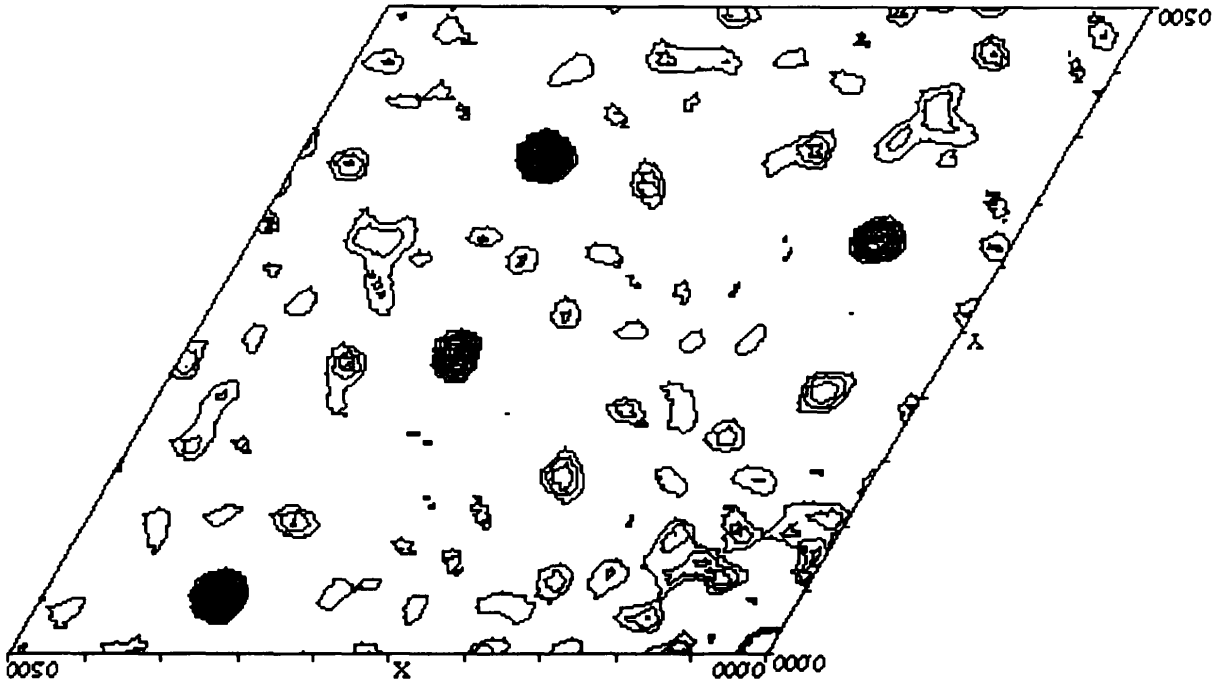


Fig. 3. Asymmetric unit of the cryocooled xenon isomorphous difference Patterson for *P*6 myoglobin, $w = 0$ Harker section. The map is contoured at 1.0σ intervals, starting at σ. Created using the *XTALVIEW*2.0 software package (McRee, 1995).

the cryocrystallographic pressure device described above should be useful for producing reasonably occupied xenon sites for biomolecules displaying similar off-rates.

One disadvantage of cryocooling is that the native and derivative data cannot be obtained from the same crystal. This may pose a problem for crystal systems that produce nonisomorphic crystals upon flash cooling. For those particular cases, room-temperature data may suffice. On the other hand, cryogenic temperatures may be required to obtain useful data and thus techniques will have to be developed for those systems whereby nonisomorphism between crystals is minimized.

5. Conclusions

We have successfully prepared a cryocooled xenon derivative of myoglobin. This method of derivative preparation avoids the increase in background and X-ray absorption from the pressurized gas as well as the hazard of pressurizing glass capillaries. This method should be of general use for proteins where the release rates of xenon are on a timescale of minutes, which is similar to or slower than that of myoglobin. Other proteins, where the off-rate of xenon is substantially faster than that observed for myoglobin, will undoubtedly require methods that allow for the flash cooling of crystals directly under xenon pressure. Such a method is currently under development in our laboratories.

This work was supported in part by NIH GM45062 of the NSF to DCR and by the DOE, Office of Basic Energy Sciences and the Office of Health and Environmental Research, and by the NIH, Biomedical Research Technology Program, National Center for Research Resources for support of the rotation camera facilities at SSRL. We thank R. P. Phizackerley and Henry D. Bellamy for helpful discussions.

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