SHORT COMMUNICATIONS

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Application of moderate hydrostatic pressure induces unit-cell changes in rhombohedral insulin

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Abstract

$2Co^{2+}$-insulin crystals were subjected to hydrostatic pressures of up to 30 bar in a nitrogen gas cell. Changes in the diffraction pattern occurred at pressures as low as 5 bar. Analysis with standard image-processing software showed unit-cell dimension changes resulting in reductions in volume of up to 2.6%.

1. Introduction

High-pressure crystallography on powders and single crystals of small molecules, to study phase transitions or compressibilities, dates back to 1933 (Cohn, 1933). Comparatively few studies have been performed on single crystals of biological macromolecules (Kundrot & Richards, 1986, 1988; Tilton, 1988; Tilton & Petsko, 1988), although owing to the relative softness of the crystals, the high solvent content and the comparatively large flexibility of most macromolecules, transitions under moderate pressure seem possible.

Previous experiments (Kundrot & Richards, 1986, 1988) on the application of very high pressures to lysozyme crystals were not designed to explore tertiary and quaternary structure changes in this monomeric tightly packed molecule. The observed structural changes were consequently very small. Studies by Tilton & Petsko (1988) on sperm-whale myoglobin at pressures of 145 atm (14.7×10^6 Pa) revealed replacement of the protein interior and a new distribution of cavity spaces. The investigation of conformational changes in more flexible proteins under pressure, including possible studies of substrate or inhibitor binding, is a logical extension of their pioneering work which could ultimately lead to the controlled creation of structural intermediates and transition states.

Rhombohedral crystals can accommodate insulin hexamers in a variety of conformations, as described previously (Derewenda, Derewenda, Dodson, Hubbard & Körber, 1989; Whittingham, Chaudhuri, Dodson, Moody & Dodson, 1996). Crystallographic and spectroscopic studies have demonstrated that the three types of insulin hexamer are structurally related and form a series (Derewenda, Derewenda, Dodson, Dodson *et al.*, 1989; Bentley, Dodson, Dodson, Hodgkin & Mercola, 1976; Baker *et al.*, 1988). Each conformation is associated with particular unit-cell dimensions as outlined in Table 1.

The hexamer in the R6 form (Derewenda, Derewenda, Dodson, Dodson *et al.*, 1989) is very tightly packed and has a smaller radius of gyration about the threefold axis than the hexameric T3R3 (Bentley *et al.*, 1976) and T6 (Baker *et al.*, 1988) structures (Balschmidt, Benned Hansen, Dodson, Dodson & Körber, 1991). Also, on average, the C_α atoms in the R6 form are 0.5 Å closer to the centre of the hexamer than in T6, as judged by an analysis of their r.m.s. distances. Thus, by applying pressure to the T6 form of rhombohedral insulin we hope to shift the equilibrium towards the more compact T3R3 or R6 states, with the possibility of trapping structural intermediates.

The Co^{2+}-insulin hexamer has been shown to undergo three different, chemically induced, transformations in solution (Thomas & Wollmer, 1989) and at least one of them, the Co^{2+}- to Co^{3+}-insulin transition, can also be performed in the crystalline state with minimal loss of diffraction (McMichael, 1996). This system is thus well suited for the exploration of the effects of hydrostatic pressure.

2. Experimental

Crystals of $2Co^{2+}$-insulin were mounted in the pressure cell shown in Fig. 1. The crystals were placed on a Mylar ledge at the centre of the cell and a few drops of mother liquor were deposited nearby. Entrance and exit windows were formed initially by mica sheets and later by Mylar foil pressed tightly against a pressure seal. The angular acceptance of the exit window was 45°, permitting, in theory, the recording of a single frame to a resolution of 2 Å at Cu $K\alpha$ wavelength. In practice, owing to the distance between the crystal and window and the rotation of the cell, the resolution is restricted to 2.5 Å. The cell has a standard goniometer fitting. Centering of the crystal in the plane vertical to the beam was performed with one slide and the spindle-axis translation. The cell does not permit reorientation of the crystal with arcs. Hydrostatic pressure was applied by compressed N_2 (oxygen free, BOC) from a standard gas cylinder fitted with a pressure reducer.

Preliminary investigations with small-angle precession photography on a sealed-tube generator showed changes in the diffraction patterns at pressures of more than 5 bar. However, as the crystals could not be aligned with a major

Table 1. *Unit-cell dimensions for different forms of rhombohedral insulin*

Crystal form	*a* (Å)	*b* (Å)	*c* (Å)	Volume (Å^3)	Volume monomer (Å^3)
$2Co^{2+}$ (T6)[a]	82.55	82.55	34.23	202009	11223
2Zn (T6)[b]	82.50	82.50	34.00	200409	11134
4Zn (R3T3)[b]	80.70	80.70	37.60	212063	11781
2Zn (R6)[c]	79.92	79.92	40.39	223417	12412
$2Co^{2+}$ (100 K)[d]	80.75	80.75	33.63	189908	10550

References: (*a*) McMichael (1996). (*b*) De Graaff, Lewitt-Bentley & Tolley (1981). (*c*) Whittingham *et al.* (1996). (*d*) Nicholson & Körber (1995).

axis along the beam, the exact nature of the changes could not be established.

Further experiments were performed on workstation 7.2 on the SRS at CCLRC Daresbury Laboratory using a rotation camera with a MAR image plate.

One set of diffraction patterns was recorded with mica windows at pressures between 0 and 30 bar (1 bar = 10^5 Pa) above atmospheric pressure. The pressure was increased in 5 bar steps. The rotation angle was 2° and the total exposure time was 60 s (experiment 1). The orientation of the crystal with respect to the beam was the same for all exposures.

Another set was recorded with Mylar windows under conditions similar to those described above. An initial exposure was taken at atmospheric pressure, then a diffraction pattern was recorded at 20 bar before obtaining a final image, again at ambient pressure (experiment 2).

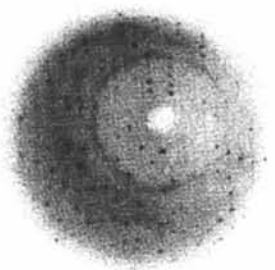
In a third experiment the crystal was initially treated as in experiment 2, *i.e.* pressurized to 1, 20 and 1 bar, but then subjected to pressures of 20 and 25 bar before being depressurized again.

The patterns were compared by visual inspection using *IPDISP* (Collaborative Computational Project, Number 4, 1994) and were also analysed quantitatively with the program *REFIX* (Kabsch, 1988).

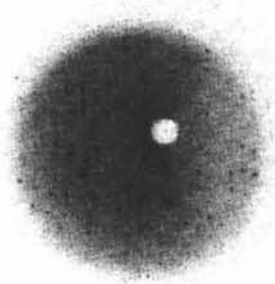
3. Results

All crystals initially diffracted beyond the maximum resolution that could be recorded (2.5 Å). In experiment 1 superposition of subsequent images showed changes at 15 bar with no loss of diffracting power. The changes persisted at 20 bar and the diffraction pattern did not change any further. Pressurization beyond 20 bar, however, resulted in a total loss of diffraction. Inspection of the crystal after the experiment revealed that the mother liquor in the cell had dried up. Analysis of the unit-cell parameters with *REFIX* showed that the space group, *R*3, was unchanged, but drastically shorter cell dimensions were obtained as shown in Table 2.

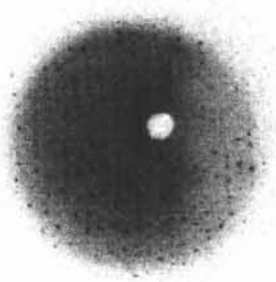
Fig. 1. The hydrostatic pressure cell viewed from the exit-window side; constructed of brass with a length of PEEK tubing (SIGMA, HPLC equipment) transmitting the pressure.



(*a*)



(*b*)



(*c*)

Fig. 2. (*a*) Rotation photograph of Co^{2+}-insulin at atmospheric pressure, with a maximum resolution of 2.5 Å. (*b*) Rotation photograph for the same orientation at 20 bar N_2 gas pressure. (*c*) Overlay of figures (*a*) and (*b*) showing a shift in the positions of many of the diffraction spots between 0 and 20 bar.

Table 2. *Unit-cell dimensions for Co^{2+}-insulin determined from experiments 1–3 using the program REFIX*

The values were calculated independently for each crystal. Note the excellent agreement at ambient pressure showing the accuracy of the cell determination. The increase in the standard deviation for the unit-cell parameters of crystal 1 supports the theory that this crystal was disintegrating due to drying out.

Experiment	Pressure (bar)	$a = b$ (Å)	c (Å)	Volume (Å^3)	Percentage change a	c	V
1	1	82.55 (2)	34.23 (1)	202009 (79)			
	5	**81.97 (2)**	**34.04 (1)**	**198075 (78)**	**0.70**	**0.56**	**1.95**
	10	80.43 (5)	33.83 (2)	189526 (174)	2.57	1.17	6.18
	15	79.93 (28)	33.38 (18)	184687 (1171)	3.17	2.48	8.57
2	1	82.55 (2)	34.23 (1)	202009 (79)			
	20	**81.89 (2)**	**34.15 (1)**	**198328 (78)**	**0.80**	**0.23**	**1.82**
	1	81.89 (2)	34.15 (1)	198328 (78)	0.80	0.23	1.82
3	1	82.55 (2)	34.23 (1)	202009 (79)			
	20	**81.84 (2)**	**33.92 (1)**	**196751 (77)**	**0.86**	**0.91**	**2.60**
	1	81.84 (2)	33.92 (1)	196751 (77)	0.86	0.91	2.60
	20	81.84 (2)	33.92 (1)	196751 (77)	0.86	0.91	2.60

In experiments 2 and 3 a significant change in the diffraction pattern was observed immediately upon pressurization to 20 bar. The change persisted when the crystals were depressurized. In experiment 3 the crystal lost its diffracting power completely at 25 bar; this was not regained when the crystal was depressurized. As a result of the use of Mylar windows, which do not leak, the crystals stayed moist throughout the latter experiments. Typical diffraction patterns are displayed in Fig. 2.

Analysis of the unit-cell dimensions using the program *REFIX* revealed that, at 20 bar, the space group is unchanged but the dimensions of a and b are shorter by approximately 0.7 Å, as shown in Table 2. The length of c changes typically by less than one half of this amount. Owing to the small r.m.s. deviation in spot position of 0.2 mm, the unit-cell dimensions could be determined from *REFIX* with high accuracy. The values derived from experiments 2 and 3 are almost identical, although the change in c is somewhat larger for crystal 3.

4. Discussion and conclusions

Although the results from experiment 1 cannot be used for a definite quantitative analysis of the behaviour of the crystals under pressure, because the exact moment of drying out of the crystal could not be established, it gave a clear indication that for this batch of crystals changes in the diffraction pattern occur at pressures above 5 bar.

The change in cell dimensions observed in experiment I goes far beyond those seen when the moist crystals are pressurized (Table 2) or cryo-cooled (Table 1). Baker *et al.* (1988) point out that the crystal packing in dry insulin crystals differs from that in wet crystals by a rotation of the hexamers about the threefold axis by several degrees concurrent with an improvement of the close packing of the molecules.

The marked increase in the standard deviations of the unit cell at 10 bar in experiment 1 indicates that the combined effects of drying out and pressure are responsible for the observed changes. One can speculate that the associated structural changes are not identical to those observed for the application of pressure alone, as performed in experiments 2 and 3. Otherwise, it should be possible in the latter experiments to pressurize the crystals beyond 20 bar and achieve a reduction in the unit-cell parameters without loss of diffraction comparable to those observed with drying out. Further well controlled experiments are necessary to ascertain whether the changes upon pressurization of the crystals are gradual as with drying out or occur abruptly in stages as we suspect now.

Experiments 2 and 3 confirmed the existence of a 'window' between 5 and 25 bar where the crystals diffract with a changed pattern. Above 25 bar diffraction power is lost irretrievably. Upon depressurization the crystals do not return to their original cell parameters.

This behaviour is surprising in that it contradicts previous experiments on the precession camera which indicated the return of the crystal to its old structural parameters when depressurized after an initial pressurization. Currently, we do not have any explanation for this difference in behaviour of our crystals. We can clearly exclude crystallization conditions, incorrect pressure measurement, cell leakage and partial drying out as possible causes. Although experiments 2 and 3 demonstrate very good repeatability further investigations under well defined conditions are desirable.

In all the experiments the crystallographic symmetry is maintained, indicating concerted changes either in the hexamer packing or in the insulin hexamer's conformation. Wollmer has modelled the collective nature of the T–R transition of the insulin trimer with targeted energy minimization (Engels, Jacoby, Krüger, Schlitter & Wollmer, 1992). Changes in the crystal which leave the crystallographic symmetry intact are compatible with this model.

Against expectation the tighter R6 structure has a larger unit-cell volume than the T6 form (Table 1) when the transitions have been induced chemically. Thus, it is doubtful whether pressurization which reduces the unit-cell volume can promote the transition. However, there are indications that the percentage changes in c are smaller on average than for a and b, decreasing the a/c ratio as expected for the R6 structure. This has to be investigated further with more careful measurements. Also, it is not certain that the initial stages of the T–R transition need large changes in the unit-cell parameters.

The exact nature of the changes can only be determined by collection of a full diffraction data set. For this purpose we are constructing an improved medium pressure cell with a wide angular acceptance that will permit a large rotation angle. We

shall also investigate the crystals over a longer timescale to explore the phenomenon of hysteresis and permit the investigation of non-instantaneous changes.

We have shown that pressurization of hexameric T-state insulin crystals leads to measurable changes in the unit-cell parameters and may open an avenue to induce transitions within the crystals. Further experiments with improved equipment, based on pressurizing Lindemann tubes as described previously (Tilton, Kuntz & Petsko, 1984; Vitali, Robbins, Almo & Tilton, 1991; Schiltz, Prange & Fourme, 1994), and on a variety of other crystal systems will be necessary to evaluate fully the technique described in this paper.

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