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Studies of Insulin Crystals at Low Temperatures. Effects on Lattice Dimensions, Temperature Parameters and Structure

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Measurements of the lattice dimensions of orthorhombic insulin crystals show small but systematic reductions in *b* and *c* on cooling from 21 to −13°C. The temperature-dependent variations in the intensities of some *hk*0 and all 0*kl* reflections to 3·3 Å were studied over the range 21 to −10°C. The observed intensity changes were compatible with the assumption of a large absolute reduction in the temperature parameter *B*, on cooling, of approximately 16 Å² from an initial room temperature value of approximately 35 Å². Large residual temperature-dependent changes in some structure amplitudes have been observed. The physical significance of these phenomena, and the experimental problems they present are considered.

Introduction

Protein crystals are known to suffer radiation damage when exposed to X-rays; a quantitative study of the effects of radiation damage on diffracted intensities has been made for myoglobin crystals (Blake & Phillips, 1962). It is important, therefore, to establish experimental conditions that reduce, as far as possible, the effects of radiation damage. Qualitative studies of the effects of cooling on orthorhombic heavy-atom-containing crystals of insulin from room temperature (~21°C) to 0 and to −13°C have provided clear evidence of enhanced radiation protection (Low, Chen, Berger, Singman & Pletcher, 1966). A quantitative study of the effects of cooling, in the same temperature range, on the lattice dimensions and on the diffracted X-ray intensities of insulin crystals was, therefore, undertaken. Normal metal-free orthorhombic crystals were used in this study. The lower limit, −13°C, was chosen at a temperature several degrees above that temperature at which, on slow cooling, the liquid of crystallization freezes spontaneously and destroys the crystal structure.

Experimental

All the data were collected from orthorhombic type *A* crystals of finback whale insulin citrate grown from citric acid/dibasic ammonium citrate buffer by a modification of the procedure of Low & Berger (1961). A solution of 0·035*M* dibasic ammonium citrate was added dropwise to a solution of 100 mg of insulin in 8 ml of 0·033*M* citric acid to persistent slight turbidity. The crystals were grown and stored in a cold room at 1±1°C. In this crystal form, space group $P2_12_12_1$; $a=57{\cdot}97$, $b=51{\cdot}72$, $c=38{\cdot}11$ Å, there are two molecules of insulin in the asymmetric unit, related to each other by a noncrystallographic twofold axis (Shoemaker, Einstein & Low, 1961). The crystals were mounted wet, in sealed thin-walled glass capillaries, with reservoirs of mother liquor above and below the crystal.

The X-ray data were collected using a General Electric SPG spectrogoniometer with goniostat (manual diffractometer). Intensity measurements were made by the stationary-crystal stationary-counter technique, using the procedure of Traub & Hirshfeld (1960), with minor modifications. The temperature for studies at 21±1°C was maintained by a room air-conditioning unit controlled by a thermostat within a tent of plastic sheeting which enclosed the diffractometer. The tent served to decrease the effect of ambient air currents.

For low-temperature studies, an apparatus was built which maintained a cooling system of dry nitrogen gas flowing down the axis of the glass capillary mount of the crystal for all setting angles. For these studies, the stream of cold nitrogen gas employed was produced by inserting an electric heating coil into a Dewar flask of liquid nitrogen. The temperature of the gas was controlled by the current flowing through the heating coil, and it was measured by means of a thermistor bead mounted in the gas stream near the crystal and forming one arm of a Wheatstone bridge.

The cold gas passed through a flexible insulated tube attached by a bracket to a looped extension of the χ rider. This rode the χ arc in such a way that, for all crystal settings, the gas delivery tube was coincident with the φ axis of the goniostat. The delivery tube was designed and packed in such a way that temperature fluctuations in the gas stream were reduced to less than 0·5°C. The thermistor was calibrated initially against a copper–constantan thermocouple. Temperatures could be measured to within 0·1°C. The temperature at the thermistor was not allowed to change by more than ±1°C from the desired value for any run. It is estimated, therefore, that the temperature at the crystal did not vary by more than ±2°C.

No attempt was made to measure the thermal gradient across the aperture of the delivery tube, but the gradient in the direction of the nitrogen gas flow was measured in the absence of a crystal by placing the thermistor at points along the φ axis, near to the normal location of crystal and reservoirs. The difference between the highest and lowest temperatures was found to be approximately 0·5°C. The absence of sharp thermal gradients was indicated by the very small changes in volume of the reservoirs of mother liquor, even when the crystal was cooled continuously for several hours and the χ and 2θ settings changed frequently to bring the crystal into a reflecting position. The glass capillary and crystal both have low thermal capacity and further, they did not interfere appreciably with the flow of cooling gas. There were no problems with icing of the capillary, even on days of high atmospheric humidity.

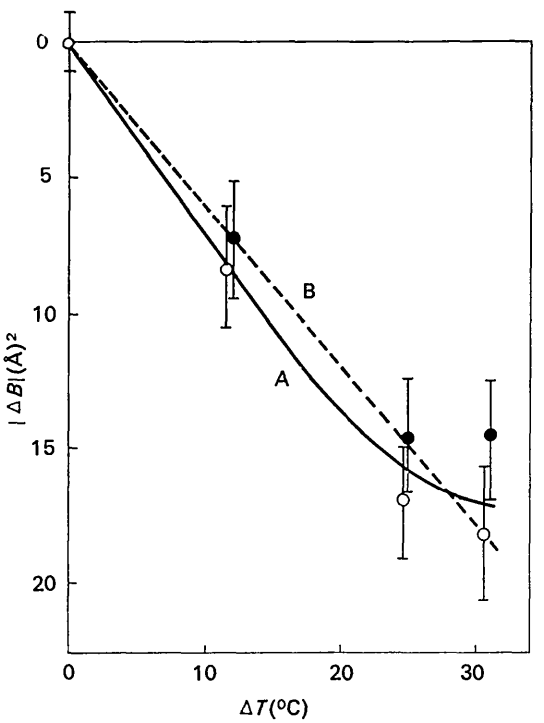

Fig. 1. The change in temperature parameter, $|\Delta B|$, as a function of the temperature difference ΔT. Solid circles represent the results of scaling the low temperature data to the room temperature data ($\Delta B<0$); open circles represent results after scaling room temperature data to low temperature data ($\Delta B>0$). The estimated standard deviation in $|\Delta B|$ values are shown.

Temperature dependence of the lattice parameters

The lattice parameters of four different crystals from the same preparation were measured over the range 21 to −13°C by determining the peak positions of the most intense axial reflections. The method of measurement was chosen to ensure that the systematic errors involved in the measurements remained constant at all temperatures in the range. Thus, changes in lattice parameters could be established more accurately than the lattice parameters themselves. The results are shown in Table 1. The temperature-dependent changes in lattice parameters recorded are not very large in comparison with the probable errors in their determination. While there is no evidence for a decrease in the a dimension on cooling, there is evidence of systematic decreases in the b and c dimensions.

Temperature-dependent X-ray intensity changes

A preliminary study of 14 $hk0$ reflections at 0°C and then at 21°C from one crystal showed changes in intensities, on warming the crystal, largely compatible with a simple increase in B (the apparent Debye–Waller temperature parameter) of approximately 7·5 Å² for a 21°C rise in temperature. The value of B for these crystals at room temperature is 30 to 35 Å² (Shoemaker *et al.*, 1961).

Exceptionally large changes in intensity incompatible with this simple interpretation were observed for the two reflections 0,14,0 and 1,14,0. Moreover, the direction of the change for the reflection 0,14,0 is inappropriate; this very strong reflection is reduced on cooling.

Another, more extensive, study of the $0kl$ intensities from all planes with spacing greater than 3·3 Å was then undertaken. With one crystal, these 140 reflections were measured at +10, −4 and −10°C. This crystal was accidently exposed to air before intensity data could be collected at room temperature; another crys-

Table 1. *Unit-cell dimensions* (Å)

T (°C)	Crystal	a	$\sigma(a)$	b	$\sigma(b)$	c	$\sigma(c)$
21	1	58·05	0·07	51·70	0·04	38·09	0·04
0	1	57·90	0·04	51·54	0·02	37·98	0·04
21	2	57·95	0·04	51·73	0·02	38·12	0·02
0	2	57·96	0·01	51·61	0·02	37·99	0·02
21	3	57·96	0·01	51·77	0·08	38·13	0·01
−5	3	57·90	0·04	51·51	0·04	37·97	0·03
−13	3	57·91	0·05	51·47	0·02	37·96	0·01
21	4	57·91	0·04	51·67	0·03	38·12	0·02
				Mean values			
21	1,2,3,4	57·97	0·07	51·72	0·06	38·11	0·03
0	1,2	57·93	0·04	51·58	0·04	37·98	0·04

tal was, therefore, used. Throughout the study, the crystals were taken several times through the cooling-warming cycle; the measured intensities at any one temperature were unaffected by the thermal cycling.

For two sets of data at temperatures t_1 and t_2, values of the scale factor s and of the differential temperature parameter, ΔB, were determined by minimizing the function,

$$\sum_k \sum_l \left\{F_{t_1}(0kl) - sF_{t_2}(0kl) \exp \frac{(\Delta B \sin^2\theta)}{\lambda^2}\right\}^2 = \sum_k \sum_l (\Delta F_{t_1 t_2})^2 \quad (1)$$

using the method of least squares.

The low temperature data were scaled to the high temperature data, and *vice versa*, to produce two values of s and of ΔB for each $\Delta T = \pm(t_1 - t_2)$. The average values of ΔB obtained are listed in Table 2. The estimated standard deviation in ΔB values, approximately 3 Å^2, includes possible contributions that arise from differences in lattice dimensions of different insulin crystals from the same batch, and possible differences in systematic errors when measurements are made on different crystals.

Table 2. *Mean values of ΔB obtained from least-squares scaling process between data collected at high and low temperatures*

t_{high}	t_{low}	ΔT	ΔB (Å)^2
21	10	11	7·8
21	−4	25	15·8
21	−10	31	16·4
10	−4	14	8·3
10	−10	20	8·9
−4	−10	6	1·0

The values of s obtained when data for the same crystal were scaled with respect to each other fell within the range $1 \pm 0{\cdot}04$ (maximum scatter). For data from the two different crystals, values $s = 1{\cdot}18 \pm 0{\cdot}06$ (maximum scatter) and $s = 0{\cdot}87 \pm 0{\cdot}07$ were found.

Pairs of the values for $|\Delta B|$ between temperature data and data at 0 and −10°C are shown plotted against ΔT in Fig. 1. At 21°C ($\Delta T = 0$) a value of ΔB of zero is shown; repeated measurement at room temperature validates the small standard deviation associated with this point. That curve which appears to fit the plotted points best shows a large rate of change of B with temperature at or near room temperature, with a fall-off to a lower rate of change as the crystal is cooled. The maximum value of dB/dt is 0·7 Å^2.°C^{-1}; the minimum is 0·3 Å^2.°C^{-1}. Because of the large standard deviations it is also possible to assume a linear relationship, with a value of 0·6 Å^2.°C^{-1}.

In order to distinguish between the curve A and the line B, it would be necessary to make intensity measurements at approximately −30°C. This cannot be done, as the liquid component of the crystals freezes and, consequently, disrupts the structure at temperatures below approximately −20°C. In contrast with carboxypeptidase-A (Quiocho & Richards, 1964) and lysozyme (Haas, 1968) it has not been possible by using alcohols, glycol and/or crosslinking reagents to modify the insulin crystals to permit cooling at this temperature without inducing gross disorder and/or increased mosaic spread (Low *et al.*, 1966).

At the maximum rate of change of B with temperature (0·7 Å^2.°C^{-1}), the Debye parameter would change from approximately 35 Å^2 at room temperature to zero at or near −40°C. Even the minimum rate of change observed within this temperature range is abnormally high and incompatible with the expected variation of the Debye parameter B, with temperature (Lonsdale & El Sayed, 1965).

For approximately 44% of the intensities measured, the variable B was adequate to account for observed differences in structure amplitudes at different temperatures. The residuals for the remaining reflections were significantly greater than their estimated standard deviations and, in general, increased in magnitude as the temperature range (t_1 to t_2) was increased. This is shown in Fig. 2, where R, defined as $\sum|\Delta F_{t_1 t_2}|/\sum|F_{t_1}|$ is plotted as a function of ΔT. The form of the plot is not intended to suggest that R is a function of ΔT independent of the specific temperatures t_1 and t_2 which define the increment. The data available are insufficient to establish the relationship between R, t_1 and t_2.

It was shown by calculations using only those reflections with small residuals ($\Delta F_{t_1 t_2}$) that the reflections for which the residuals were large did not dominate the least-squares estimate of either ΔB or s.

Among the reflections with significantly large residuals $\Delta F_{t_1 t_2}$ are several which exhibit strong dependence between $\Delta F_{t_1 t_2}$ and ΔT, as shown in Fig. 3. For other reflections, Table 3, where the residuals were generally smaller although still an appreciable fraction of observed room temperature amplitudes, no particular dependence on ΔT was observed.

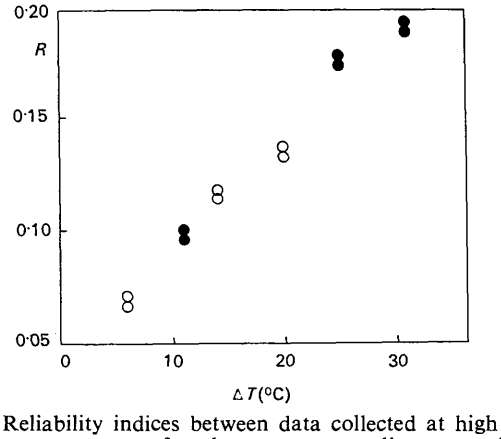

Fig. 2. Reliability indices between data collected at high and low temperatures after least-squares scaling operations. Closed circles correspond to calculations for which either t_1 or t_2 was room temperature. For the open circles, neither t_1 nor t_2 was room temperature. At each value of ΔT, both values of R are plotted.

Table 3. *Some ΔF values for selected reflections*

Results are given for reflections in which the ΔF values, while significantly larger than their standard deviations, show no particular dependence on ΔT. All data are on an approximately absolute scale; figures in parentheses represent estimated standard deviations in ΔF values.

	ΔT (°C)			
hkl	11	26	31	$\|F\|$ abs
0,12,0	−32 (6)	−16 (6)	−23 (6)	517
0,14,4	−36 (8)	−52 (8)	−53 (8)	460
0, 1,5	27 (4)	42 (4)	43 (4)	326
0, 2,6	14 (5)	17 (5)	11 (5)	249
0, 2,7	22 (6)	20 (6)	36 (6)	198

The magnitude of some of the observed changes should be noted. Thus, on an absolute scale,* the reduction in structure amplitude for the strong reflection 0,14,0 on cooling through 31°C is 220 electrons. The structure amplitude of the strong reflection 075 is approximately 263 electrons at room temperature.

* The structure factor amplitudes for the room temperature data were put on an approximately absolute scale by comparison with values for another preparation of crystalline whale insulin. The scale for these latter data was determined experimentally (unpublished studies) by a comparison of the measured intensities from a protein crystal and from a crystal of sodium chloride, both of known volume.

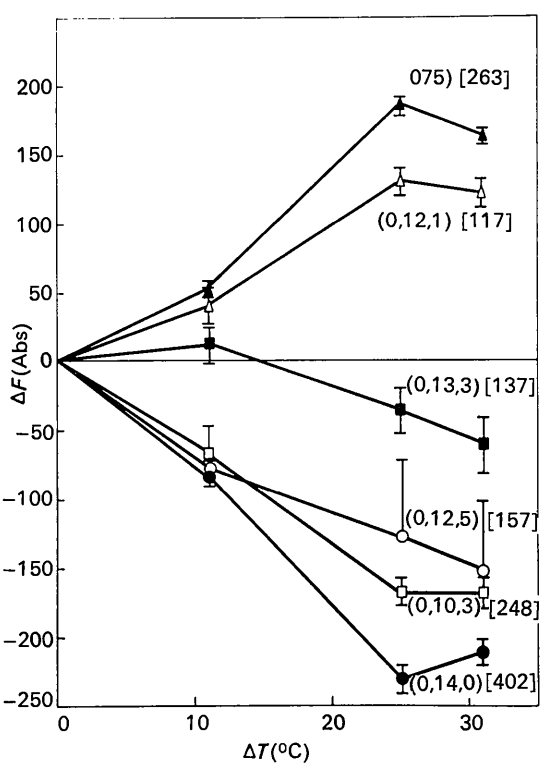

Fig. 3. Values of the residuals ΔF_{t1t2}, on an absolute scale, for selected reflections that show a strong dependence of ΔF_{t1t2} on ΔT. Figures in square brackets are the room temperature structure amplitudes on an absolute scale.

Discussion

The temperature-dependent changes in lattice parameters recorded are not very large in comparison with the probable errors in their determination. While there is no evidence for a decrease in the *a* dimension on cooling, there is evidence of systematic decreases in the *b* and *c* dimensions over the range 21 to −13°C. The approximate value 120×10^{-6} of linear thermal expansion coefficient for the insulin crystals is of the same order of magnitude as the values found for ice (LaPlaca & Post, 1960) and for anthraquinone (Lonsdale, Milledge & El Sayed, 1966).

The residual changes in the $|F|$ which cannot be associated with reduction in the apparent temperature parameter may perhaps be associated with simple lattice parameter changes in a contracting structure. Such an explanation would demand that the transform of the cell contents and/or of the molecule changes rapidly in certain regions.

An attempt was made to determine whether the changes in lattice parameters are formally large enough to account for the magnitude of these effects. From the absolute values for the salt-free structure amplitudes of the *h0l* hemoglobin reflections at several shrinkage stages (Perutz, 1954), the maximum slope of the transform was estimated. Near the 12,0,1 reflection, the slope is approximately 85×10^3 e per r.l.u. and near the $20\bar{1}$ reflection 93×10^3 e per r.l.u. These values correspond to the steepest slopes, with the exception of the region near 001, where the slope is approximately four times more steep than at any other place.

The change, $|\Delta F|$, in the 0,12,1 reflection of insulin over the temperature range, 21 to −10°C, is approximately 125 electrons. If it be assumed that this reflects only a change in lattice dimensions, and thus a sampling of the unit-cell transform at a point, then the change is essentially dependent on the shrinkage in the *b* axis dimension. For this reflection, the implied slope of the transform is 83×10^3 e per r.l.u. The order of magnitude of the effect is not, therefore, unreasonable. However, the relevance of this comparison depends on the assumption that the form of the insulin transform is essentially similar to that of hemoglobin. This has not been demonstrated.

The thermal contraction hypothesis may account for the lattice parameter changes and for the magnitude of some of the residual ΔF values. It offers no explanation however, for the apparent large absolute reduction in the apparent temperature parameter over the range studied.

An alternative hypothesis would attribute these effects to structural changes in the crystal between room temperature and −10°C. During the transition state, the rate of change of *B* with temperature may be abnormally large, and there are alterations in the slope of the Debye factor curve (Lonsdale & El Sayed, 1965). The transition state hypothesis could also account for the change in lattice parameters and the large changes in intensity on cooling.

Protein crystals contain a large volume of water (for insulin, approximately 54% by volume), most of which, in proteins of known structure, behaves as liquid water surrounding the protein molecule so that there are few direct protein–protein contacts (myoglobin: Kendrew, Dickerson, Strandberg, Hart & Davies, 1960; lysozyme: Blake, Mair, North, Phillips & Sarma, 1967; ribonuclease A: Kartha, Bello & Harker, 1967; ribonuclease *S*: Wyckoff, Hardman, Allewell, Inagami, Tsernoglou, Johnson & Richards, 1967;tosyl-α-chymotrypsin: Matthews Sigler, Henderson & Blow, 1967; subtilisin: Wright, Alden & Kraut, 1969). In myoglobin, which has been investigated in great detail, there are a few molecules of water bound to the polar groups at the molecular surface (Kendrew, 1962). Profound changes occur in the ordered regions of liquid water on warming from 0° C to 25° C. Furthermore, temperature changes would affect the structure promoting tendencies of sidechain groups of protein molecules (Frank 1965; Nemethy & Scheraga, 1962). Although it is not possible to define a precise model of the structural changes to be expected, the system appears one in which changes might well occur, particularly at the protein water interface.

The large change in the *B* parameter might be considered simply as evidence of disorder effects when a crystal, grown in the cold, is warmed to room temperature, except that the intensity changes are maintained through several thermal cycles, and there is much evidence that protein crystals, once disordered, are not reordered in a cyclic process.

If a marked reduction in the thermal vibrations were a dominant feature of the transition from crystal at room temperature to crystal at −10° C, this should be evident in the appearance of diffraction maxima beyond the normal limit of minimum spacing. No conclusive experimental evidence has been found for a general increase in average intensity for insulin beyond 3·3 Å. In contrast, we note that King (1958), in a qualitative study of the effects of cooling on the X-ray diffraction pattern (photographic data) of crystalline ribonuclease observed a significant (15%) reduction in minimum spacing.

Although the observations reported here do not permit an unambiguous explanation, they do clearly emphasize two points of experimental importance in the collection of intensity data. First, that measurements should be made at a temperature which does not fall within the range of large temperature-dependent changes in intensity, and that the temperature should be carefully controlled during data collection. Secondly, all observations suggest that data collection at low temperatures may have a considerable advantage, although it cannot be correlated simply with an overall reduction in the apparent *B* parameter.

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