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Low-Temperature Protein Crystallography. Effect on Flexibility, Temperature Factor, Mosaic Spread, Extinction and Diffuse Scattering in Two Examples: Bovine Trypsinogen and Fc Fragment

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Abstract

The crystal structure of bovine trypsinogen in 50% methanol–water mixture has been refined to an R of 19·5%. It shows no significant differences to the trypsinogen crystal structure in magnesium sulphate. The crystal structure in 67% methanol–33% water mixture at 213 K shows two extra water molecules, but no electron density in the region of flexible segments. There is a substantial effect on the physical properties of these crystals at low temperature. The temperature factor, mosaic spread and diffuse scattering are substantially decreased. The primary and secondary extinctions are considerably increased. These parameters change rapidly in a narrow temperature range, indicative of a phase transition. The transition temperature depends on the solvent composition. Low-temperature studies on Fc-fragment protein crystals show similar behaviour.

Introduction

The trypsinogen–trypsin system has been studied in great detail and various crystal structures have been solved and extensively refined at the highest resolution allowed by the crystalline order (Huber & Bode, 1978). These studies have indicated large disordered segments in the proenzyme trypsinogen which become ordered in the active enzyme trypsin. Flexibility starts rather abruptly. Four segments are disordered: the N terminus to Gly 19, Gly 142 to Pro 152, Gly 184 to Gly 193 and Gly 216 to Asn 223. These segments constitute the activation domain and form a tightly interdigitating structural unit in the active enzyme trypsin. Several residues of this domain are directly involved in substrate binding. The term flexibility in protein structures has been discussed in a recent article by Huber (1979). Flexibility may be due to thermal motion, conformational heterogeneity or lattice defects involving the molecules as a whole. These situations will produce similar effects on the electron density, either smearing it out over a large volume or distributing it over several sites, but with a low weight. A decision as to which situation holds might be provided by spectroscopic studies or low-temperature X-ray crystallographic analysis. Thermal agitation can be substantially reduced by conducting the diffraction experiments at low temperature; the remaining disorder is then largely due to conformational heterogeneity. We report here low-temperature X-ray crystallographic studies on trypsinogen and Fc-fragment protein.

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Crystal structure at room temperature

Bovine trypsinogen crystals were grown at 293 K from 1·5 mol dm^{-3} magnesium sulphate solution (pH 6·9) containing trypsinogen and pancreatic trypsin inhibitor at a molar ratio of 4/1. They are normally stabilized in 2·4 mol dm^{-3} magnesium sulphate (pH 6·9) and it was in this salt-rich mother liquor that the crystal structure of this proenzyme was solved earlier (Bode, Fehlhammer & Huber, 1976; Fehlhammer, Bode & Huber, 1977). The crystals were transferred to 50% (v/v) methanol–water mixture (0·03 mol dm^{-3} cacodylate, 0·03 mol dm^{-3} $CaCl_2$, pH 7·9 before addition of the methanol) *via* Ficoll (Pharmacia) at 277 K. No damage to the crystals was observed.

Three-dimensional X-ray intensity data up to 1·8 Å resolution were collected on a rotation camera. About 12 060 unique reflexions (66% of the possible reflexions to 1·8 Å resolution) were measured with significant intensity and evaluated (Schwager, Bartels & Jones, 1975).† Based on the final model of native

† Lists of structure factors and coordinates for the room-temperature and 213 K data have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 34822 (70 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

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trypsinogen (Fehlhammer, Bode & Huber, 1977), the structure was refined to an R of 23·1%, using procedures described (Huber, Kukla, Bode, Schwager, Bartels, Deisenhofer & Steigemann, 1974; Deisenhofer & Steigemann, 1975). The model was further improved by correcting a few bad contacts with the solvent molecules through a graphics display system (Jones, 1978) and then several cycles of refinement were carried out by Deisenhofer, using his modified version of the energy-refinement program by Jack & Levitt (1978). This reduced R to about 19·5% (in a resolution range of 1·8 to 6·5 Å) and improved the model considerably. A difference-Fourier map calculated at this stage between native trypsinogen and the present structure showed no significant electron density and the regions of flexible loops showed no residual density indicating no influence of the alcohol solvent on the flexible loops in particular and the overall structure in general. Trypsinogen crystals analysed in polyethylene glycol solvent had allowed similar conclusions to be drawn (Bode & Huber, 1978).

Low-temperature-analysis experimental set-up

Data were collected by photographic methods. The rotation camera and the X-ray tube with a copper target and a graphite monochromator are installed in a cold room. The temperature in this room can be varied from 293 to 183 K. The influence of temperature on the primary beam and the film response has been determined. In general, photographic films increase in speed with increasing temperature, although in many cases no effect was detectable (Morgan, 1944; Schlesinger, 1979). We used OSRAY T4 X-ray films in our investigations and examined the response of these films over the temperature range 263 to 213 K. The blackening curves were determined at three temperature steps. The input power of the X-ray generator and other relevant conditions were kept strictly identical. The intensity of the primary beam was reduced by using an additional attenuator (0·1 mm copper + 0·05 mm brass). Two films in each case were exposed in a time series at 261, 243 and 213K, and the response curve was determined. As seen from Fig. 1, these response curves are superimposable. The absorption factor, due to the first film, is 2·38, 2·36 and 2·37, respectively, at 261, 243 and 213 K. These observations provide evidence that the sensitivity of these films to X-rays over this range of temperature does not change. Also, the primary beam, monochromator and X-ray camera are not affected within this temperature range.

Crystal structure at 213 K

To cool the trypsinogen crystals to temperatures in the 203 K range, it was necessary to use a higher methanol concentration (67%) to avoid freezing of the crystals (Douzou, Hui Bon Hoa, Maurel & Travers, 1976). Starting from 273 K, the rotation intensity photographs were made at 273, 258, 243, 233, 228, 223, 218 and 213 K as we gradually cooled the room. We compared the distribution of intensities between the photographs made at room temperature and at reduced temperatures. Up to 243 K, no difference was observed. At 233 K, there were some differences in the distribution of intensities. The differences increased substantially as the room was further cooled to 213 K. At this temperature the intensity data were recorded. About 7500 independent reflexions were obtained. A difference electron-density map between the low-temperature and room-temperature crystals was calculated, using the refined phases of the room-temperature structure factors. In general, this map appeared rather noisy. The two highest peaks (about 4σ) could, however, be easily interpreted as solvent molecules. One solvent molecule is frozen around Ile 63 and Asn 74. It does not appear to be hydrogen bonded and the shortest non-bonded distance is 4·5 Å. Another solvent molecule is found close to Lys 87, Ser 88 and Asp 153. It forms a hydrogen bond with Ser O^{γ} (2·6 Å). All the non-bonded contacts are greater than 3·12 Å. There is no interpretable high residual density in regions of the flexible segments. This shows that cooling has not influenced the flexible residues.

In the analysis at 213 K, we noted that the cell dimensions shrank by about 1%. We did not observe radiation damage to the crystals at this temperature.

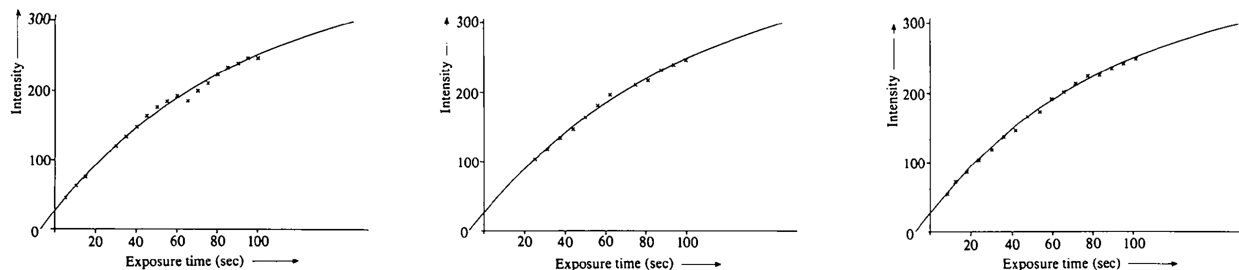


Fig. 1. Plots showing the film response at three temperatures: (*a*) 261 K; (*b*) 243 K and (*c*) 213 K.

Temperature factor

Thermal motion of atoms causes a decrease in the scattering factor by a factor $\exp[-B(\sin^2\theta/\lambda^2)]$, where $B = 8\pi^2\bar{U}^2$ and $\bar{U}^2$ is the mean-square displacement of the atoms from their mean positions along the normal to the reflecting planes (Debye, 1914). Apart from thermal motion, the temperature factor also takes into account static disorder. It has been deduced by Mössbauer absorption measurements that a substantial part of the temperature factor in a protein crystal is due to static disorder (Parak & Formanek, 1971).

The refined structure of trypsinogen at room temperature (as described earlier) has shown an overall B factor of 13·3 Å^2. At 213 K, we obtained $B = 6{\cdot}1$ Å^2. As mentioned earlier, we have collected about 2500 independent intensity data at each intermediate temperature. These data have been scaled to the room-temperature data, using a scale factor and a relative temperature factor as variables. An unexpected and interesting trend has been observed in terms of the B factor and scale factor. The scale factor behaviour will be discussed later under extinction. It remains constant over a large temperature range (273 to 238 K) and then shows a sharp drop over a narrow range (238 to 233 K) with a further slow decrease to 213 K.

This experiment has been repeated twice with trypsinogen crystals. Slightly different methanol concentrations have been used to study the influence of solvent composition. It was further extended to Fc-fragment protein (human) (Deisenhofer, Colman, Epp & Huber, 1976), to acquire generality of this observation. Fig. 2 illustrates the behaviour of the B factor against cooling temperatures. All four cases exhibit a strikingly similar pattern. In addition, these results demonstrate a shift in phase transition towards lower temperature with increased methanol content in the solvent mixture. Fc fragment in 40% methanol shows this transition between 240 and 243 K. Trypsinogen in 67% methanol undergoes this change around 233 K, whereas in 70% and 75% methanol the transition temperatures are 228 to 223 and 213 to 208 K, respectively.

The values of B were also determined at similar steps when the temperature was elevated from 198 to 273 K. These values turned out to be approximately the same as before, thus showing reversibility and no hysteresis within the time range of these experiments (several days).

The results of the first experiment with 67% methanol will not be used. In this case, the exposure time was frequently varied and hence the parameters dependent on input conditions cannot be reliably treated. The remaining three cases will be discussed simultaneously.

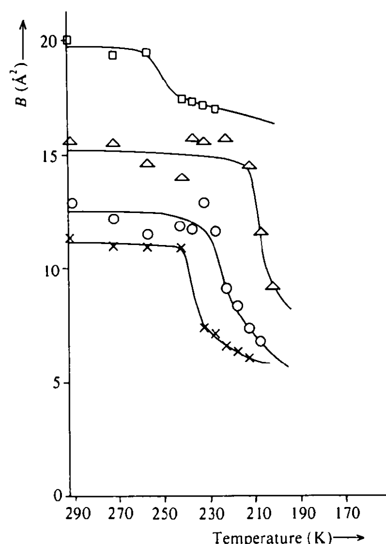


Fig. 2. Plots of B *vs* temperature in various solvent concentrations: Trypsinogen in 67% methanol + 33% water (×), 70% methanol + 30% water (○), 75% methanol + 25% water (△), and Fc fragment in 45% methanol + 55% water (□).

Diffuse scattering

Diffuse scattering originating from the protein crystal arises due to the various types of disorder. It is generally regarded as an unwanted effect but can provide useful information about many physical properties of the crystal.

Fig. 3 shows the comparison of rotation photographs of trypsinogen and Fc fragment before and after the phase transition. The background around a number of reflexions has been estimated at various cooling temperature steps. These data have been plotted against the temperature and are illustrated in Fig. 4. The zero background value has been determined from photographs made without the crystal in the capillary; this value is indicated in Fig. 4 by a dashed line.

Clearly, the reduction in background scattering is substantial. The trend in reduction follows that observed for B. In general, this observation is evidence that the order of the protein crystals increases substantially.

Spot size and mosaic spread

Yet another effect of cooling was observed in terms of a large reduction in spot size. We expected that this must follow the same trend outlined earlier for the temperature factor and background blackening. We, therefore, quantitatively estimated the reflexion size in terms of raster points (1 raster = 0·05 mm) by printing the reflexion images. The same reflexions were picked up for estimation at various temperatures. A plot of change in spot size against cooling temperatures is shown in

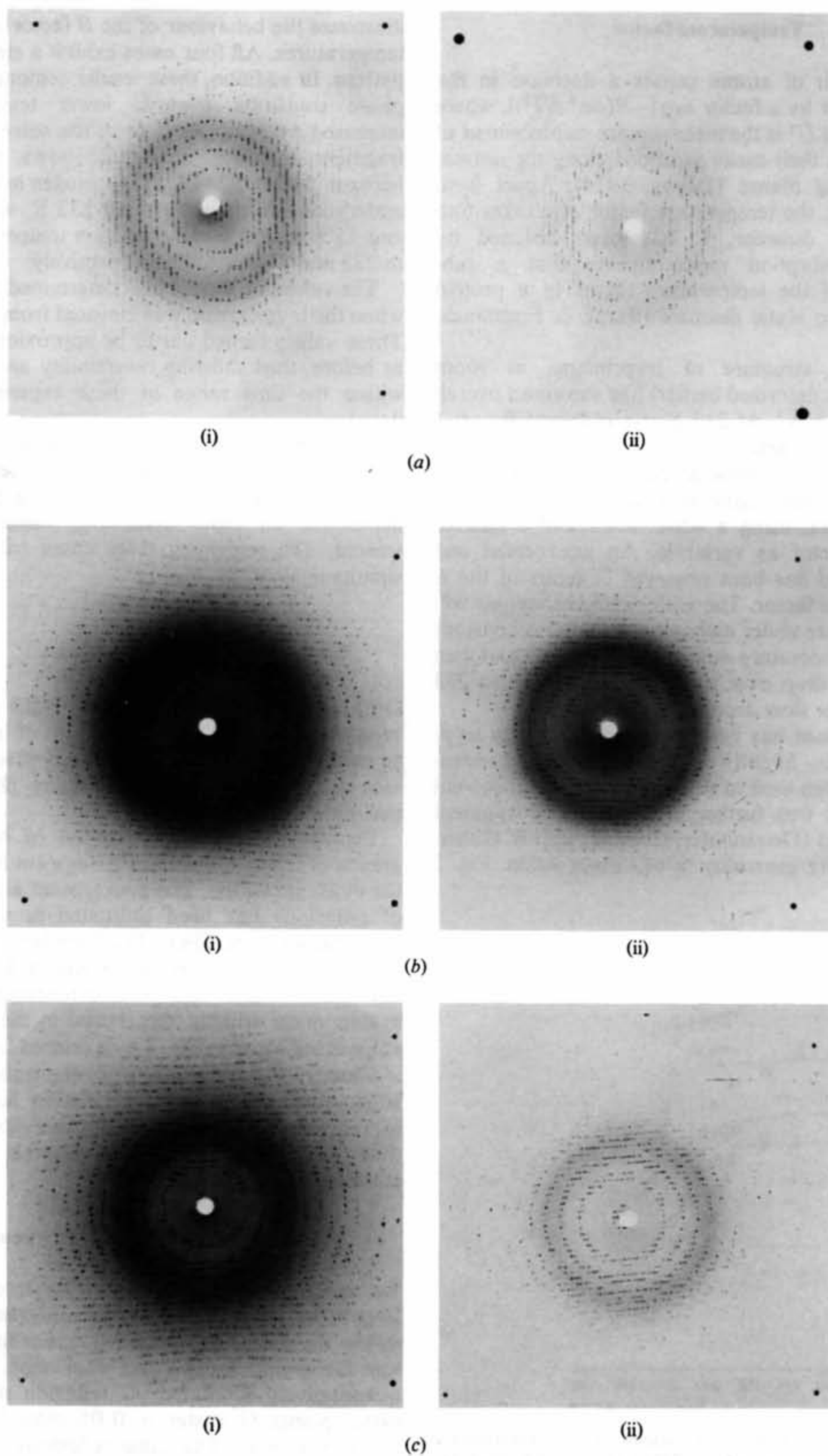
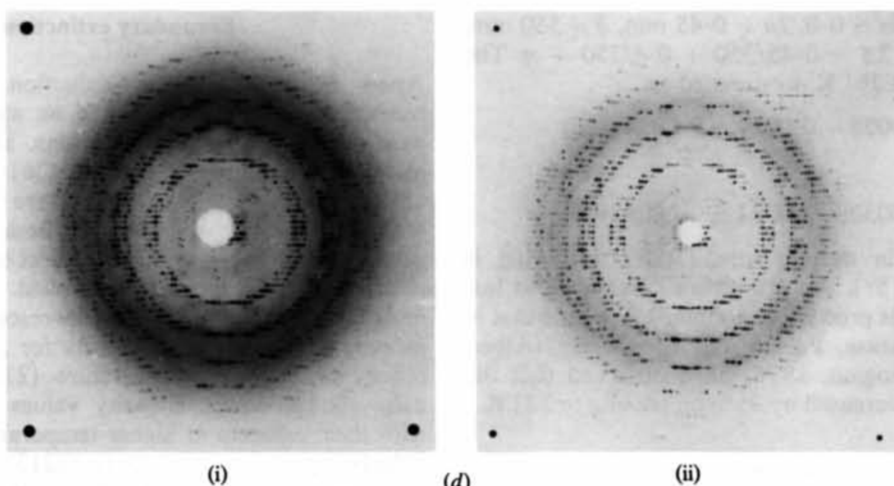


Fig. 3. X-ray intensity photographs before (i) and after (ii) the transition range: Trypsinogen in (*a*) 67% methanol + 33% water; (*b*) 70% methanol + 30% water; (*c*) 75% methanol + 25% water.



(*d*)

Fig. 3 (*cont.*) X-ray intensity photographs before (i) and after (ii) the transition range. (*d*) Fc fragment in 45% methanol + 55% water.

Fig. 5 and follows exactly the phase change observed for the other parameters.

This observation of reduction in spot size is a consequence of a change in mosaic spread.

To verify this, we made two series of stationary photographs – one before the phase transition (261 K) and another after (213 K). These photographs were made at an interval of 0·03° covering an angular range of about 0·6°. In order to minimize the dispersion spread of the $K\alpha$ doublet, low-angle reflexions were selected. Reflexions of or close to the zero layer line were used. About 7–8 reflexions were followed on the photographs from appearance to disappearance. At 261 K, we obtained a full width of 0·33 ± 0·03° and similar measurements at 213 K gave 0·18 ± 0·03°. Trypsinogen crystals in 70% methanol were used for this analysis.

The analytical formula (Arndt & Willis, 1966) to calculate the angular range over which a crystal diffracts under equi-inclination conditions as it rotates through the Bragg position is given by

$$2\Delta = \delta c + \delta_f + \eta + \delta\sigma,$$

where δc = angle subtended by the crystal at X-ray tube focus
= $2a/s$, where $2a$ is the linear dimension of the crystal and s the distance between focus and crystal.

δ_f = angle subtended by the X-ray tube focus on the crystal
= $2f/s$, where $2f$ is the linear dimension of the focus.

η = mosaic spread of the crystal.

$\delta\sigma$ = dispersion spread.

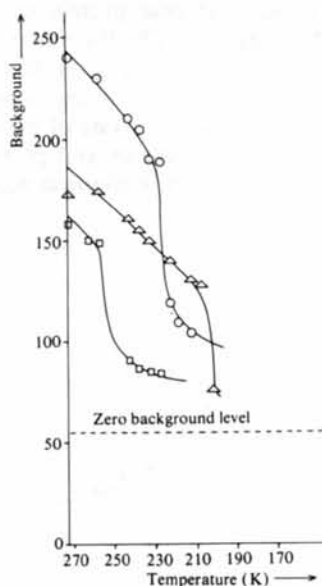


Fig. 4. Plots of background blackening *vs* temperature: Trypsinogen in 75% methanol + 25% water (△) and 70% methanol + 30% water (○), and Fc fragment in 45% methanol + 55% water (□).

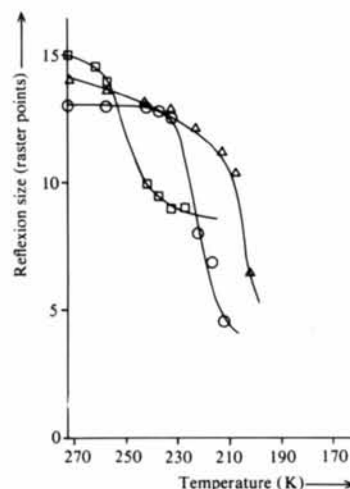


Fig. 5. Plots showing the changes in reflexion size on cooling: Trypsinogen in 75% methanol + 25% water(△) and 70% methanol + 30% water (○), and Fc fragment in 45% methanol + 55% water (□).

In our case, $\delta\sigma \simeq 0{\cdot}0$, $2a = 0{\cdot}45$ mm, $\delta = 350$ mm, $2f = 0{\cdot}4$ mm, $2\Delta = 0{\cdot}45/350 + 0{\cdot}4/350 + \eta$. The mosaic spread at 261 K is estimated as

$$\eta_1 = 0{\cdot}0058 - 0{\cdot}0024 \simeq 0{\cdot}0034 \text{ (rad)}$$

and at 213 K as

$$\eta_2 = 0{\cdot}0032 - 0{\cdot}0024 \simeq 0{\cdot}0008 \text{ (rad)}.$$

The reduction in mosaic spread due to cooling is 0·0026 rad (0·15°). We are aware that only this last difference value is precisely determined. We note that in the case of elastase, Petsko and co-workers (Alber, Petsko & Tsernoglou, 1976) have observed that the mosaic spread decreased by 25% on cooling to 223 K.

Primary extinction

For primary extinction in an ideally perfect crystal, the net result is that the intensity of the diffracted beam is proportional to $|F|$ rather than $|F|^2$ for the ideal mosaic crystal. Integrated reflexions for perfect crystals are therefore much weaker than for imperfect crystals.

In our present experiment, the mosaic spread at low temperature is reduced to a quarter of its room-temperature value; it is therefore expected that primary extinction is influenced to an appreciable extent. Primary extinction must appear in terms of the scale factor when the identical data sets from low- and high-temperature crystals are individually scaled to the common source. This way, we obtain a scale factor of about 1·5 between the two sets.

As mentioned before, all low-temperature data sets were individually scaled to the native crystals and the scales thus obtained have been plotted in Fig. 6. It provides another document for the observed systematic effects.

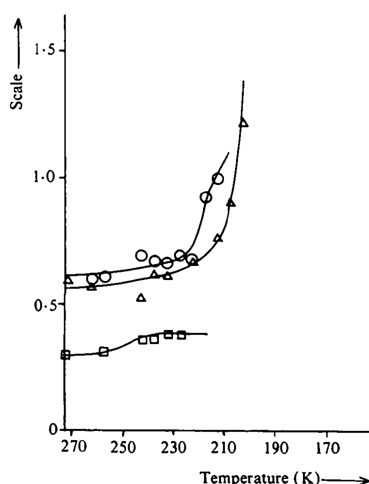


Fig. 6. Plots showing the scale-factor behaviour on cooling the crystals: Trypsinogen in 75% methanol + 25% water (△) and 70% methanol + 30% water (O), and Fc fragment in 45% methanol + 55% water (□).

Secondary extinction

Apart from the general reduction in intensity of reflexions, we further observed an attentuation in the case of low-angle strong reflexions. The intensity data at two temperatures, one before (261 K) and one after (213 K) the phase transition, were first individually scaled to the native crystals. These data were then scaled to each other so that the effect of individual scale and temperature factors is removed. About 80 of the most intense reflexions in the low-resolution range were picked up from both data sets for comparison. The reflexions at lower temperature (213 K) systematically showed lower intensity values when compared with their partners at higher temperature (261 K). On calculating the ratio $F(261)/F(213)$, we obtained a value of 1·04. Similar calculations for 35 less-intense reflexions (these were in the accurately measurable range) within the same resolution limits gave a ratio of about 0·91.

We note from the present analysis that this effect is not impressively large, despite the substantial reduction in mosaic spread. This could be due to the fact that the intensities diffracted by a protein crystal are generally weak.

Discussion

The reduction in temperature factor and the alignment of mosaic blocks have jointly influenced the background scattering and extinction. All parameters measured, B, mosaic spread, diffuse scattering and extinction, indicate a phase change. This phase change is observed in such diverse protein crystals as trypsinogen and Fc fragment. The transition temperature is strongly solvent dependent. Higher methanol content shifts the transition region to lower temperature (Fig. 7). The phase change is to a state of higher order. It is unclear whether this reflects crystal properties only or also indicates a reduction in thermal motion or in the

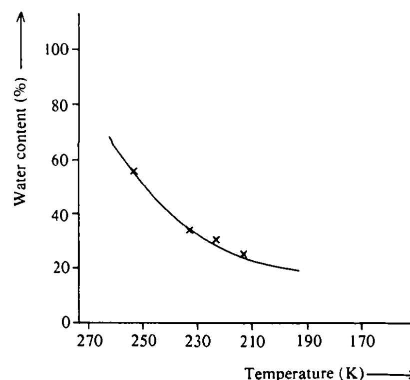


Fig. 7. Plots of percentage water content in mixed solvents *vs* transition temperatures.

amount of conformational heterogeneity of the protein molecules, if there is any. In the literature, we could not find any indication of the changes of the physical parameters of methanol–water mixtures in the temperature range. The phase change observed reflects changes in the whole crystal system composed of the protein molecules and the solvent channels. The dependence on solvent composition might indicate that there is a composition-dependent phase separation on the solvent in the channels between the protein molecules which in turn could affect the ordering of the molecules and the relative alignment of the mosaic blocks.

The difference Fourier map at 213 K showed only two additional solvent molecules. As described earlier, there is no significant residual electron density in the region of the flexible segments. Cooling to 213 K is without observable effect on these segments. On the other hand, the overall temperature factor is already reduced to less than half of its room-temperature value (13·3 to 6·1 Å^2). Mössbauer spectroscopic investigations on shock-frozen myoglobin crystals at liquid-nitrogen temperature have shown that the temperature factor reduced to about one half of the room-temperature value (10·42 to 5·0 Å^2) (Parak & Formanek, 1971).

From these experiments, we may not expect a further large reduction in B between 213 and 103 K, but it is difficult to exclude the possibility that shock-freezing increases crystal disorder.

The absence of electron density for the disordered segments in trypsinogen at 213 K may be interpreted as conformational heterogeneity (microstates), but we are not able to exclude the possibility that the thermal mobility of these segments is so extensive that they remain invisible even after a substantial reduction in thermal motion. The 'visibility' limit may be around 50 Å^2.

It should be noted that the difference electron-density map between room-temperature crystals and low-temperature crystals below the transition is rather noisy. The substantial changes in mosaic spread, diffuse scattering and extinction of the low-temperature phase compared with room-temperature crystals may be causing this effect. The phase change is towards an ideal crystal. In fact, every protein crystallographer intending to work at subzero temperatures with methanol–water solvent mixtures should be aware that such phase transitions can occur and should try to avoid such conditions.

It is a pleasure to thank Dr E. Weber for his continuous help throughout the experiment as well as for many interesting discussions and Dr J. Deisenhofer for his energy-refinement calculations. One of us (TPS) is grateful to the Humboldt Foundation for a fellowship. We would also like to thank Mrs E. Preuss for drawings and Mr E. Mock for maintaining the apparatus in the cold room.

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