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Insulin. Some Shrinkage Stages of Sulfate and Citrate Crystals

By J. Ralph Einstein and Barbara W. Low

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A.

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Orthorhombic crystals of both insulin sulfate and insulin citrate give discrete shrinkage stages under controlled drying conditions. The stages observed are described and some observations made concerning their interrelationships. One shrinkage stage appears to provide evidence about the symmetry of the insulin dimer.

Introduction

When a wet protein crystal, grown from aqueous solution, is removed from its mother liquor, it loses water and the crystal shrinks. When it is in equilibrium with its mother liquor, it is in the 'wet' form. The 'air-dried' form of the crystal (a rather ambiguous term) is that form consequent to prolonged exposure to the atmosphere of the laboratory. Crystals of some proteins have been shown to exhibit several discontinuous lattice changes on drying from the wet to the air-dried state (Perutz, 1946; Huxley & Kendrew, 1953; Drenth, 1957). That is, if the relative humidity of the atmosphere with which the crystal is in contact is reduced in a controlled manner, several discrete shrinkage stages may be observed. This phenomenon has now been observed with orthorhombic insulin crystals. Preliminary X-ray data for some shrinkage stages of these crystals are presented below. Evidence they provide concerning the symmetry of the insulin dimer is discussed.

Experimental

Procedures used in growing bovine insulin sulfate and citrate crystals have been described elsewhere (Low & Richards, 1954; Low & Berger, 1961). Several slightly modified procedures have also been employed. The existence of the shrinkage stages for these crystals was first demonstrated by continuous drying experiments very similar to those carried out by Perutz (1946) with hemoglobin. The method employed by Perutz as reported by Huxley & Kendrew (1953) was to permit a crystal, mounted as usual in a thin-walled glass capillary tube, to lose water slowly to the atmosphere through a small hole pierced in the seal of the tube. In our experiments, a short length of narrow-bore polyethylene tubing (i.d. 0·011″, length about 2″) was sealed with wax into the open end of a thin-walled glass capillary, so as to provide a fine leak for the capillary. A wet crystal was mounted in the glass capillary through the open end; then, after excess mother liquor had been removed, this end was sealed and the capillary mounted on the precession camera. While the crystal dried, a continuous series of X-ray diffraction patterns were obtained with a low (5°) precession angle, and without the use of a screen. A series of distinct diffraction patterns were observed. Certain films showed two distinct sets of reflections, corresponding to successive crystal forms in the drying process. Streaks connecting pairs of spots were not observed. Attempts were made to halt the drying of the crystal, in order to maintain a single shrinkage stage, by sealing the open end of the polyethylene tubing. These efforts were not very successful, perhaps because of temperature fluctuations near the crystal.

A device was then designed and built (Einstein, 1958) to maintain a crystal in contact with an atmosphere of constant relative humidity during X-ray photography with the Buerger precession camera. With this device, a crystal can be equilibrated with an aqueous salt solution of known relative humidity. This general method of humidity control has been used to investigate shrinkage stages by other investigators (Huxley & Kendrew, 1953; Drenth, 1957). By using a series of saturated solutions of different salts, it is possible to cover at fairly small intervals the entire range of relative humidity (O'Brien, 1948). The device used in these experiments differed from those used by the other investigators. Some comparative discussion of the various instruments is given elsewhere (Einstein, 1961). This device employs a small, light-weight, thermostatted enclosure fixed to the goniometer head of the precession camera. The crystal is mounted in a glass capillary, which is sealed into the lid of a small cell containing the salt solution; the cell is mounted within the enclosure. The enclosure is provided with two mylar windows, which permit passage of the incident and diffracted X-ray beams. The temperature of the enclosure and of its contents is maintained at 25 °C. (slightly above room temperature) by an electrical heater winding and an electronic thermostat. It was possible, using this device, to maintain a single shrinkage stage during the several days required for X-ray photography. An improved model of this device, differing in minor

Table 1. *Preliminary X-ray data for orthorhombic bovine insulin crystals*

Form	Anion	State	a	b	c	V	$d_{min.}$	Experimental conditions
A	Sulfate	Wet	58·0 Å	51·5 Å	38·5 Å	115,500 Å^3	2·1 Å	In mother liquor
B	Sulfate	Wet	46·7	51·9	44·6	108,000	1·7	In mother liquor
A′	Sulfate	Shrinkage stage	53·8	53·8	35·4	102,500		C.D.*; over satured solution of KH_2PO_4†
B′	Sulfate	Shrinkage stage	46·7	51·9	41·6	101,000	3·5	C.D.; over satured solution of Na_2SO_4‡
C	Sulfate	Shrinkage stage	51·8	53·3	34·1	97,000	3·0	C.D.; over satured solution of Na_2SO_4
P	Sulfate	Air-dried	44	51·4	30·4	69,000	5·5	C.D.; rapid air drying
S	Sulfate	Air-dried	47	54	32	81,000		C.D.
A	Citrate I	Wet	58·2	51·9	38·5	116,300	2·2	In mother liquor
A	Citrate II	Wet	58·1	51·85	39·1	117,800	2·2	In mother liquor
C′	Citrate II	Shrinkage stage	51·1	53·4	35·5	97,000		C.D.; over satured solution of Na_2SO_4
R	Citrate II	Air-dried	47	53	33	82,000		C.D.

The space group of all forms except C is $P2_12_12_1$, $Z=4$. The space group of form C is $I2_12_12_1$, $Z=8$.
The precise lattice dimensions of the crystals in the wet state depend on the conditions of crystallization (p_H, salt concentration) and may differ by $\pm 0{\cdot}5$ Å. The lattice dimensions of some of the shrinkage stages may differ by about the same amount.

* C.D.—Continuous drying experiment.
† Relative humidity (25 °C.) approximately 97%.
‡ Relative humidity (25 °C.) approximately 93%.

constructional details from the one used in these experiments, has been built (Einstein, 1961).

Preliminary X-ray data for all shrinkage stages of orthorhombic insulin crystals investigated so far are given in Table 1. Published data for the wet and air-dried crystal forms (Low, 1952; Low & Shoemaker, 1959; Low & Berger, 1961) are included in the Table for comparison.

The unit cell dimensions of wet insulin crystals (sulfate and citrate) depend upon both the detailed preparative procedures used for a given batch and on the age of the preparation. The dimensions of the wet forms of the crystals studied were not measured precisely in these experiments. To have done so would have involved prolonged exposure of the crystal prior to the shrinkage study.

Crystal forms observed during drying

When a wet insulin sulfate (type *A*) crystal dries, it does not exhibit all the shrinkage stages nor both of the air-dried forms for insulin sulfate. Continuous drying experiments have demonstrated two alternative shrinkage pathways:

(1) A → B′ → P, and (2) A → A′ → C → S .

Observations suggest that the particular pathway followed by one insulin sulfate crystal may depend on the amount of mother liquor adhering to the crystal at the start of the drying process. Thus, when crystals were wiped dry of all adhering mother liquor before further drying by evaporation, they usually followed the shrinkage pathway A → B′ → P. When a small droplet of liquid was left adhering to the crystals, they were observed usually to follow the shrinkage pathway A → A′ → C → S.

The existence of two alternative modes of shrinkage behavior may perhaps be related to differences in pH and salt concentration which may occur in the crystal during drying. If there is some mother liquor adhering to the crystal, the ions in this mother liquor may enter the crystal as the water evaporates. This phenomenon has been discussed in detail by Huxley & Kendrew (1953).

Only one shrinkage pathway has been observed with insulin citrate: A → C′ → R.*

The shrinkage stages so far investigated were all obtained at relative humidities in the range 90–100%. However, these stages represent the only discrete stages observed in the continuous drying experiments between wet (~ 100%) and air-dried.

Discussion

There are many similarities between the crystal forms, and some other general features of interest. Certain similarities between the wet A and B forms and the air-dried P form have been discussed elsewhere (Low & Shoemaker, 1959).

(1) All the values for b are distributed over a relatively very narrow range. The value for the wet forms (A) is less than that for any other form except P, i.e. the shrinkage stages correspond to expansion of the cell along this axial direction.

(2) The a value for the wet (A) form is the maximum value observed. The c value of the wet (A) form is an intermediate value.

(3) The forms B and B′ are similar in unit-cell

*) *Note added in proof*: During further studies with insulin citrate crystals from another batch the shrinkage pathway A → C was observed.

dimensions and strikingly similar in the intensity distribution of the $hk0$, $h0l$ and $0kl$ diffraction maxima. A similar relationship exists for the forms C and C′.

(4) There are marked similarities between the broad features of the intensity distributions of the reflections from all the forms; this is particularly evident in the $0kl$ reflections of A, B, C and P (Fig. 1).

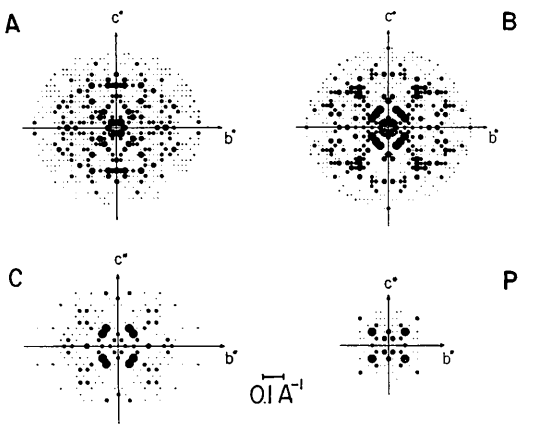

Fig. 1. ($0kl$) weighted reciprocal lattice sections for the four crystal types A, B, C, and P. The F^2 values of the various crystal forms are not on the same scale. Asterisks indicate outstandingly high values of F^2.

It thus appears very probable that the molecular shifts between these crystal forms, referred to a fixed set of axes a, b, c (with which the axes a, b, c of all the crystals are, respectively, aligned), are principally simple translations along the three axes.

(5) Type C crystals. The following reflections were collected: $hk0$, $h0l$, $0kl$ and $1kl$. The systematic absences restrict the space group to either $I2_12_12_1$ or $I222$.

We have assigned the space group $I2_12_12_1$ to these crystals on the basis of the following considerations. In $I2_12_12_1$ the three mutually perpendicular two-fold screw axes are non-intersecting, as they are in $P2_12_12_1$. In $I222$ the three two-fold screw axes intersect at a point. The transformations $P2_12_12_1 \rightarrow I222 \rightarrow P2_12_12_1$ during shrinkage would be most surprising, since they would involve two very extensive reorganizations of the packing structure. Further, the P form itself has the pseudo-symmetry $I2_12_12_1$ (Low & Shoemaker, 1959).

Symmetry of the insulin dimer

In the space group $I2_12_12_1$ there are, besides the three sets of non-intersecting two-fold screw-axes (as in $P2_12_12_1$), three sets of non-intersecting two-fold axes. If one two-fold axis (parallel to either a, b or c) is introduced at an appropriate location in the lattice of the space group $P2_12_12_1$, then the space group $I2_12_12_1$ is generated.

In the primitive unit cells of orthorhombic insulin sulfate and citrate crystals the asymmetric unit is a dimer (MW 5733×2). The dimer appears to be the stable unit in aqueous solutions acid to the isoelectric point, except under special conditions (Moody, 1944; Oncley *et al.*, 1952; Kupke & Linderstrøm-Lang, 1954). We implied earlier, as a consequence of the discussion of the similarities between the broad features of the intensity distributions, and will, with this further evidence, now assume that the dimer particle has basically an invariant structure; that the two molecules of which it is composed do not shift or shift only slightly with respect to each other during any transition considered here.

In the space group $I2_12_12_1$ there are eight asymmetric units. From the arguments above it follows, first, that in form C the two halves of the dimer must be related to each other by a two-fold axis and, second, that the point group or pseudo point group of the dimer in all the forms is 2.

Elsewhere (Shoemaker, Einstein & Low, 1961) we have presented evidence concerning the identity of this particular two-fold axis (whether parallel to a, b or c) and its approximate position in the type A crystals. Because of the limited diffraction data for type C ($d_{\min.} = 3$ Å) it would be more cautious to conclude that this axis may be only a pseudo twofold axis (pseudo $I2_12_12_1$) although this conclusion is contrary to our personal convictions (Low & Einstein, 1960).

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