THE INFLUENCE OF UREA ON CRYSTALLIZATION AND POLYMORPHISM OF HEN LYSOZYME

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1. Introduction

As many chemical studies now support the idea that enzymes may have different stable conformations, we attempted to induce them by physical and chemical means. In a previous paper [1], we pointed out the effect of temperature on the crystallization of hen egg-white lysozyme (EC 3.2.1.17), which enabled us to characterize a new orthorhombic form, called B form, quite different from the tetragonal crystals (A form) [2, 3]. The present note deals with our first results on the influence of urea. Urea is a strongly hydrogen-bonding solvent and well known as a denaturant agent by breaking up either hydrogen bonds and/or hydrophobic non polar interactions. In the case of lysozyme it has already been reported that urea has two modes of action: an instantaneous but completely reversible inhibition of its enzymic activity and a slow but irreversible action, whereby native lysozyme is transformed into closely related active substances [4, 5]. An increased activity rather than the expected inactivation was observed [6]. James and Hilborn [7] pointed out an apparent increase in enzymic activity in 1.5 to 6.0 M urea solutions and concluded "that the activation by urea results from changes induced in the conformation of the enzyme and not directly from effects upon cell walls". Quite independent experiments achieved in our laboratory [8] showed that the activity of several lysozymes was maximum in urea solutions of about 3 M. Furthermore, experiments achieved by Stark et al. [9] and Cejka et al. [10] established that the low concentrations of cyanate formed in urea solutions might react with free amino groups. Thus the observed effects, in solution, of urea on lysozyme, might be caused by cyanate. Anyway one can suspect that urea triggers changes in the tridimensional structure: hence this work on crystallization.

2. Materials and methods

A commercial sample of hen lysozyme was used throughout this research (lysozyme 6 X crystallized, lot 7107, from Miles). All other reagents were purchased from Merck or Prolabo. Our crystallization experiments were achieved at different temperatures (from $-4°$ to $60°$) as follows: to 75, 50, 35 or 10 mg lysozyme, dissolved in 0.5 ml water (or urea solution), were added 0.0625 ml 0.2 M acetate buffer, pH 4.7 (containing urea when necessary) and 0.1875 ml water (or urea solution); after centrifugation, 0.75 ml of a 10% NaCl solution (containing urea when necessary) was added to the clear supernatant followed by a drop of toluene. The crystals were analyzed by the precession camera technique.

* 92nd Communication on lysozymes.

3. Results

3.1. *At temperatures lower than 22–25°*

3.1.1. The time factor

Fig. 1 shows the crystal growth rate in 75 mg/1.5 ml solutions of lysozyme carried out at 20°. The rate was increased in presence of 0.375 to 0.75 M urea. Above these values the growth was slower and the delay in crystallization was at least six months in 3 M urea at room temp. Of course this phenomenon was even more obvious with less concentrated protein solutions: for example with 35 mg/1.5 ml it required 2 months instead of 22 hr to get crystals of the same size (about 0.5 mm long) in 0.375 to 1.125 M urea. With larger amounts of urea we have not yet obtained any crystals in 9 months at 20°. At this temperature, the pH value of the solution never exceeded 5.3 after crystallization. In attempts to crystallize in presence of larger amounts of urea, two ways were suitable: either to lower the temperature or to increase the protein concentration. Thus we obtained tiny tetragonal crystals in 5 M urea at 4° with a 5% lysozyme concentration within 8 days, and even in 7 M urea in less than 6 hr but with a largely increased protein concentration.

3.1.2. The crystalline quality

All the crystals in urea were smaller than those grown in absence of urea, much more fragile and sensitive to shocks; indeed they often presented flaws, holes, corrosion images under the microscope.

3.1.3. X-ray data

The X-ray analysis showed that all crystals were tetragonal, space group $P4_32_12$, in the experiments we have carried out. But the changes in intensities occurred very rapidly. Even with 0.375 M urea they could be seen in the 3 Å region mainly in the [001] zone. They increased as the concentration did. For example at 1.125 M, they were obvious and affected even the 6 Å region. Similar results were obtained in a 2 M urea solution. Moreover in all cases the isomorphism was retained. The lifetime of the crystals in X-ray beam seemed to be as long as the lifetime of normal ones. Despite their smallness and their optical defects, these crystals were well ordered and the diffraction maxima remained fairly intense out to a spacing of about 2 Å. X-ray investigation of the modifications occurring in the structure of lysozyme can thus easily be performed.

3.2. *At temperatures higher than 25°*

Above 25° we never obtained tetragonal but always orthorhombic crystals; high temperatures promote orthorhombic crystal growth. But experiments showed that we have to distinguish the respective role of temperature and of pH.

In a previous paper [1] we have described an ortho-

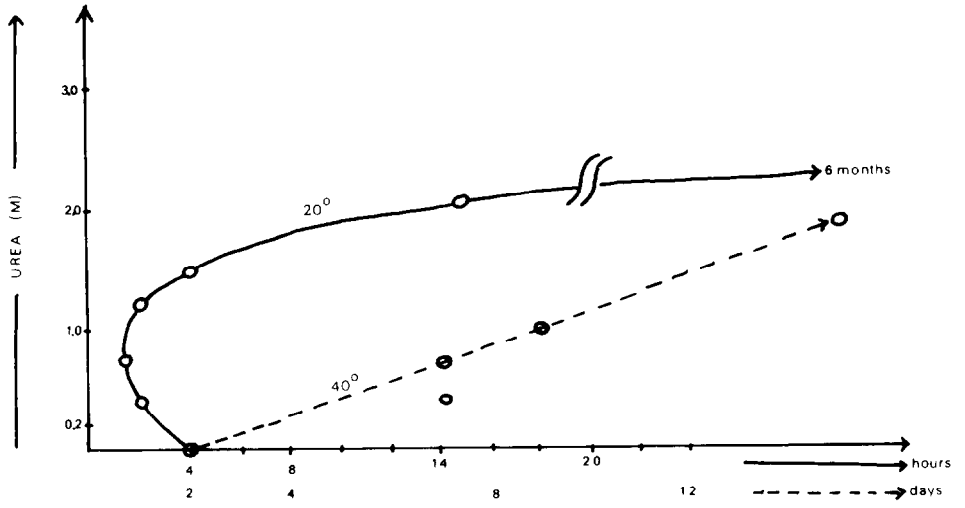

Fig. 1. Crystal growth rate of a 5% lysozyme solution as a function of the molarity of urea at 20° and 40°.

Table 1
Crystals forms of lysozyme chloride.

Medium	T°	pH	Crystal form	*a* (Å)	*b* (Å)	*c* (Å)	Space group
5% Lysozyme 5% NaCl	−4 → 25	4.7– 7.1	Tetragonal	79.1	79.1	37.9	$P4_32_12$
5% Lysozyme 5% NaCl	25 → 60	4.7– 7.4	Orthorhombic	56.3	73.8	30.4	$P2_12_12_1$
5% Lysozyme 5% NaCl	4 → 25	7.4–10	Orthorhombic	59.0	68.6	30.4	$P2_12_12_1$
5% Lysozyme 5% NaCl +0.375–7 M urea	4 → 25	4.7– 5.3	Tetragonal	79.1	79.1	37.9	$P4_32_12$
5% Lysozyme 5% NaCl +0.375–0.75 M urea	25 → 40	4.7– 6.25	Orthorhombic	56.2	73.7	30.9	$P2_12_12_1$
5% Lysozyme 5% NaCl +0.75–3 M urea	25 → 40	4.7– 7.1	Orthorhombic	56.0	66.5	30.9	$P2_12_12_1$

rhombic form occurring at temperatures from 25° up to 60°. This form, called B form, was obtained within a pH range from 4.7 to 7.1. In presence of urea the phenomenon was rather complex. At 40° the pH varied of course, becoming alkaline as a function of time and all the more as the protein concentration decreased, lysozyme buffering the medium. As far as our experiments were carried out there exists, as Palmer [2] pointed out as early as 1948, a form depending upon alkaline pH. Its lower limit seemed to be around 7.4–8 and we were unable till now to get suitable crystals above 9. Within this range, the crystals were orthorhombic, space group $P2_12_12_1$ with a = 59.0, b = 68.6, c = 30.4 Å (table 1). Obviously this orthorhombic form was different from the B form, and from Palmer's one too.

3.2.1. With rather low urea concentrations

From 0 to 0.375 M, regardless of the protein concentration (75 or 50 mg/1.5 ml) we obtained orthorhombic crystals, $P2_12_12_1$, a = 56.2, b = 73.7, c = 30.9 Å (table 1). They looked like the B form morphologically and indeed were very similar but not absolutely isomorphous, particularly the c parameter varying from 30.4 to 30.9 Å, and slight changes occurring in the intensities. The pH varied from 4.70 to 6.40 at 40°. Already here we noticed the difference in the behaviour of A and B crystals in the presence of urea: the B form was more sensitive and the isomorphism was not retained.

3.2.2. From 0.750 to 3 M urea concentrations

The pH varied more, from 4.7 to 7.1 and again we obtained orthorhombic crystals in about 20 days. Morphologically they were small elongated prisms or needle-like crystals. But the most striking feature was that though they belonged to the same space group $P2_12_12_1$, a = 56.0, b = 66.5, c = 30.9 Å, they were quite different from the two others (table 1). With urea concentrations higher than 3 M, we observed a precipitate; until now we have not found the right conditions for crystallization.

3.2.3. An interesting problem was to elucidate what happened with preformed A crystals in urea. From 0 to 1.125 M urea, the transition phenomenon occurred again and we obtained the same crystals as those grown directly at 40°.

4. Discussion

If urea did not prevent crystallization it was not a good additive as it is in many ionic salts solutions. In almost all cases it delayed the growth rate, at low or high concentrations. Below 22–25°, we always obtained A crystals in urea solutions up to 7 M. In the experiments we have carried out (until 2 M), the isomorphism was retained but the intensities were changed and all the more affected as the urea concentration increased, as if we were in presence of a continuous phenomenon. Above 25°, urea included drastic changes in the B form; the crystals were no longer isomorphous, and as soon as 0.75 M urea, lysozyme crystallized in a new orthorhombic form different from the others. Of course, it remains to be seen if all these forms are related to each other and how. Already we know that the transition phenomenon is not abolished in presence of urea. Moreover, as biochemists have pointed out that the activity of lysozyme is quite different in presence of urea, we have to check the reactivity of the crystals against substrates or analogs.

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