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Orthorhombic Lysozyme Solubility

BY FELECIA EWING

Science and Technology Corporation, Madison, AL 35758, *USA*

ELIZABETH FORSYTHE

Universities Space Research Association, Huntsville, AL 35806, *USA*

AND MARC PUSEY*

NASA/MSFC, Space Science Laboratory, Biophysics ES 76, *Huntsville, AL* 35812, *USA*

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Abstract

The orthorhombic, or high-temperature, form of chicken egg-white lysozyme typically appears at temperatures ≥ 298 K. Solubility diagrams have been determined for this form of lysozyme from pH 4.0 to 5.4 in 0.2 pH increments using the micro-column technique. Data were collected in the 297–317 K temperature range which resulted in phase diagrams similar in overall shape to those obtained for the lower temperature tetragonal form. Specifically, the solubility increased with increasing temperature and decreased with increasing precipitant concentration. However, the solubility of the orthorhombic form is considerably less sensitive to temperature than the tetragonal form, resulting in a more flattened slope. On the other hand, pH effects on the high-temperature form were opposite to those on the low-temperature form. When holding the precipitant concentration constant, the solubility decreased with increasing pH for the orthorhombic form. Previous tetragonal data were incorporated with these orthorhombic data to produce intercept values. These values varied with both pH and precipitant concentration, but the general tendency of the slope was to decrease with increasing pH.

Introduction

Chicken egg-white lysozyme is the standard test material used to investigate protein crystal growth and nucleation (Ataka & Asai, 1988; Durbin & Feher, 1986; Howard, Twigg, Baird & Meehan, 1988; Pusey & Gernert, 1988). The majority of these studies used the tetragonal form, with the orthorhombic form occasionally being used. The crystal growth behavior of orthorhombic lysozyme apparently contrasts sharply with that of the tetragonal form, having the ability to grow large crystals (> 1 cm) with no growth cessation or flow sensitivity (Nyce & Rosenberger, 1991; work in progress). These differences make the orthorhombic form an invaluable comparison with the tetragonal form in protein crystal growth studies. Serious research into orthorhombic lysozyme nucleation and crystal growth requires an accurate phase diagram, which until now has not been available.

Macromolecules are sensitive to their environment, this sensitivity often being reflected by conformational flexibility and chemical variability. This makes the crystal growth of biological macromolecules different from that of small molecules. Previous studies have shown that at temperatures above 298 K tetragonal lysozyme crystals undergo a phase change to the orthorhombic or physiological form (Jollès & Berthou, 1972). This change is manifested in the structural and catalytic properties of lysozyme. Evidence of these changes was acquired by means of ^{13}C nuclear magnetic resonance spectroscopy (Cozzone, Opella, Jardetzky, Berthou & Jollès, 1975). Thermodynamic studies have shown a sharp break in the Arrhenius plot indicative of these changes in the catalytic properties (Saint-Blancard, Clochard, Cozzone, Berthou & Jollès, 1977). Also, the high-temperature form of lysozyme's optimal activity was 1–2 pH units lower than that of the lower temperature form (Saint-Blancard, Maurel, Constant, Berthou & Jollès, 1981). Finally, after cooling of warmed lysozyme solutions, the persistence of the nucleation and growth of orthorhombic crystals in the tetragonal phase region indicated a conformational change (Berthou & Jollès, 1974). However, all structural differences between the orthorhombic and tetragonal forms are minimal when comparing their actual conformations by X-ray crystallography (Berthou, Lifchitz, Artymiuk & Jollès, 1983).

* To whom correspondence should be addressed.

Herein, we report on the orthorhombic lysozyme solubility diagram obtained from 2–7%(*w/v*) NaCl and from 298–313 K, pH 4.0–5.4. Other than temperature and crystal form, these are the same conditions as previously used for tetragonal lysozyme. Thus, we are able to determine the temperature for the orthorhombic→tetragonal phase change as a function of solution pH and temperature.

Materials and methods

All non-protein materials were reagent grade. The buffer was 0.1 *M* acetic acid, adjusted to final pH with saturated NaOH after addition of the appropriate amount of NaCl. All pH measurements were determined at room temperature. To minimize pH changes, 0.1 *M* buffer concentrations were used. Lysozyme concentrations, after dilution into dH_2O, were determined using A (1%, 281.5 nm) = 26.4 (Aune & Tanford, 1969).

Chicken egg-white lysozyme (Sigma) was prepared for solubility column packing (Cacioppo, Munson & Pusey, 1991; Pusey & Gernert, 1988; Pusey & Munson, 1991) by recrystallization in 0.1 *M* sodium acetate pH 4.6, 3%(*w/v*) NaCl. Initially, the protein was dialyzed against acetate buffer and the recrystallization was conducted at 310 K to ensure the orthorhombic crystal form. This form required concentrations ≥ 100 mg ml^{-1} to grow a sufficient crop of crystals for column packing. Visual inspection of the crystals was used to verify the orthorhombic form. Equilibration solutions for the columns were prepared by dialyzing the supernatant from the initial crystallization against the desired final salt concentration and pH at 310 K. After packing, each column was equilibrated with the appropriate solutions and solubility determinations were measured as previously described (Cacioppo *et al.*, 1991; Pusey & Gernert, 1988; Pusey & Munson, 1991).

Results

The micro-column method allowed minimal use of protein with the maximum amount of data returned for an extended solubility diagram determination (Pusey & Munson, 1991). Orthorhombic lysozyme crystals for column packing were more difficult to batch crystallize than its low-temperature counterpart due mainly to the higher protein concentrations required. Typically, crystals grown for packing were equilibrated at 0.1 *M* NaAc, pH 4.6, 3% NaCl and 310 K. As previously noted, orthorhombic crystals were virtually impossible to grow without a mixture of tetragonal crystals at pH 4.0 (Cacioppo & Pusey, 1991). The temperature range for data collection was from 297 to 317 K, although only that within the 298–313 K range is reported. Data collection was discontinued at 317 K because of the appearance of amorphous precipitate in the reservoir solutions. The lower limit for data collection was fixed at 297 K due to the orthorhombic→tetragonal phase transition. Most of the 2% NaCl data gave erratic results and required several repetitions. No clearly observable reason for this difficulty could be found but one reason may be that at 2% NaCl one borders on the 'optimal' crystallization conditions for orthorhombic lysozyme (George & Wilson, 1994).

In overall shape, the phase diagrams for the orthorhombic crystal form imitated the lower temperature results reported previously (Cacioppo & Pusey, 1991). Fig. 1 shows a representative orthorhombic data set at pH 4.6. This surface plot illustrates the effects of NaCl (precipitant) concentration and temperature on the equilibrium solubility. The solubility increased with increasing temperature and decreased with increasing salt concentration. The overall solubility of the orthorhombic form was considerably less sensitive to temperature than the tetragonal form, resulting in a more flattened slope. The solubility data for all data collected at each pH and salt concentration were fitted to a second-order polynomial and the expansion coefficients obtained are listed in Table 1. These coefficients can be used to calculate the solubility for orthorhombic lysozyme at any point over the range investigated between 298 and 313 K.

Three surface plots are shown in Fig. 2 which demonstrate the effects of pH on orthorhombic lysozyme. Figs. 2(*a*), 2(*b*) and 2(*c*) are surface plots at 3, 5 and 7% NaCl, respectively. Each plot displays the x and y axis of increasing pH from 4.0 to 5.4 and temperature from 298 to 313 K. Note the

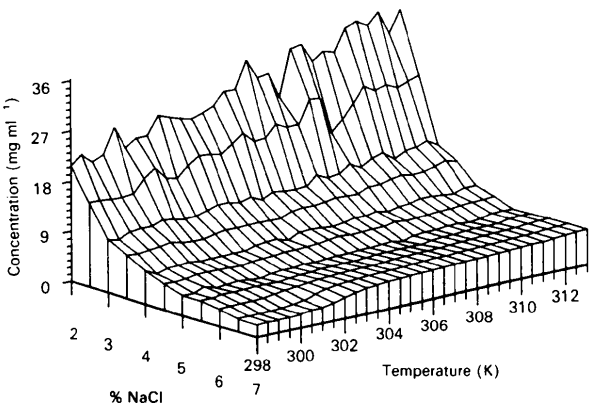

Fig. 1. Surface plot of the solubility for orthorhombic lysozyme, pH 4.6, 0.1 *M* NaAc buffer. This plot illustrates the effects of NaCl concentration and temperature on the equilibrium solubility. The trends shown are typical of the solubilities obtained for all pH values. Data points were collected at irregular temperature intervals and were interpolated to fit a grid at regular temperature and salt concentrations.

Table 1. *Coefficient values to determine orthorhombic lysozyme solubilities*

The solubility data fitted to a second-order polynominal using least squares gave these coefficients that are valid over the temperature range 298–313 K (25–40 °C). Using the following equation $A+BX+CX^2$, where X = temperature in °C, one can interpolate the solubility for orthorhombic lysozyme. For example, at pH 4.6, 3% NaCl and 30 °C, the solubility would be 10.8 mg ml $^{-1}$.

pH	% NaCl	A	$B\times10^2$	$C\times10^3$	Average % deviation
4.0	2.0	122.6	−494.1	94.94	2.99
4.0	3.0	19.69	−81.29	23.73	4.65
4.0	4.0	4.637	−9.178	7.811	3.81
4.0	5.0	12.12	−56.84	12.21	4.19
4.0	7.0	5.04	−18.58	4.424	4.26
4.2	2.0	48.5	−136.0	35.26	3.83
4.2	3.0	3.155	48.87	−0.6517	3.43
4.2	4.0	6.93	−13.62	6.503	4.07
4.2	5.0	9.165	−32.54	7.145	5.06
4.2	7.0	6.663	−27.46	5.386	4.40
4.4	2.0	31.27	−102.6	29.00	4.67
4.4	3.0	16.99	−58.31	13.84	4.23
4.4	4.0	10.67	−46.77	11.01	6.15
4.4	5.0	0.9406	11.19	0.1417	4.60
4.4	7.0	1.875	−3.003	1.879	4.99
4.6	2.0	8.702	23.31	10.99	6.53
4.6	3.0	10.67	−24.48	8.301	3.96
4.6	4.0	9.203	−37.79	9.017	5.57
4.6	5.0	2.54	−7.435	4.227	5.75
4.6	7.0	2.738	−7.466	2.573	6.94
4.8	2.0	21.77	−81.93	23.35	6.90
4.8	3.0	10.75	−45.35	11.89	4.97
4.8	4.0	4.442	−13.23	4.855	5.11
4.8	5.0	5.625	−25.52	5.964	4.90
4.8	7.0	2.758	−9.848	2.595	5.88
5.0	2.0	11.51	−16.09	12.45	5.61
5.0	3.0	13.50	−60.66	12.92	5.18
5.0	4.0	2.867	−3.612	2.756	4.72
5.0	5.0	3.548	−12.41	3.503	5.35
5.0	7.0	2.331	−7.414	1.987	4.65
5.2	2.0	75.85	−444.7	76.56	10.3
5.2	3.0	2.081	6.890	2.329	7.55
5.2	4.0	3.697	−10.55	3.544	5.28
5.2	5.0	2.827	−8.772	2.824	5.55
5.2	7.0	2.208	−6.692	1.758	4.56
5.4	2.0	30.97	−159.7	33.07	8.48
5.4	3.0	15.09	−84.87	17.42	14.8
5.4	4.0	6.666	−31.55	6.983	5.51
5.4	5.0	4.704	−22.37	5.088	5.66
5.4	7.0	2.550	−9.340	2.161	9.63

differing scales used on the z, or solubility, axis. The solubility decreased with increasing pH for each salt concentration represented here. In contrast, tetragonal lysozyme showed decreasing solubility with pH at 3% NaCl, approximately equivalent solubilities with pH at 5% NaCl, and increasing solubilities with pH at 7% NaCl (Cacioppo & Pusey, 1991).

The solubility data collected for tetragonal and orthorhombic lysozyme in this laboratory provided sufficient resources to calculate the phase-change temperatures. Using the coefficients determined previously for tetragonal lysozyme and those reported here for orthorhombic lysozyme, we were able to calculate the intercept values for these two forms as a function of pH and NaCl concentration. Fig. 3 displays the point at which solubilities are equal at pH 4.6 and 3% NaCl. In this case, the phase transition occurs at 295 K. This figure also shows the difference in the slopes which represent the response of solubility to temperature. Note that the low-temperature tetragonal slope is definitely greater than the high-temperature orthorhombic slope. Previous data collected at pH 4.0 showed that the orthorhombic solubility diagram retained its comparatively level slope below 298 K, while tetragonal solubility curves became progressively steeper above 298 K (Pusey & Gernert, 1988). These equi-solubility values were a function of pH as well as precipitant concentration. Fig. 4 demonstrates that the general tendencies of the intercept values are to decrease with increasing pH. The precipitant concentration effects did not follow readily observable trends.

Discussion

The phase diagram of orthorhombic lysozyme was determined using the same solution conditions as previously used for the tetragonal, or low-temperature, crystal form. The main experimental

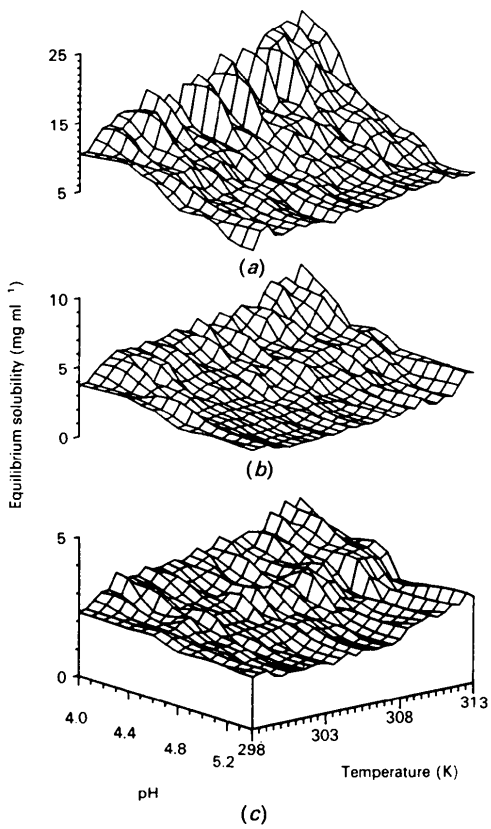

Fig. 2. Surface plot of pH effects on orthorhombic lysozyme solubility. (*a*), (*b*) and (*c*) represent the precipitant concentrations of 3, 5 and 7% NaCl, respectively. Note that the concentration axes are different for all three graphs.

differences were the temperature range covered and the preparation of the crystals. The micro-column method developed in this laboratory enabled us to rapidly collect closely spaced solubility data over a broad range of conditions (Cacioppo *et al.*, 1991; Pusey & Munson, 1991). The data for each set of conditions were fitted to second-order polynomial equations that provide a table for easy calculation of orthorhombic saturation concentrations over the range investigated.

Several laboratories have previously reported initial solubility measurements for orthorhombic lysozyme. The few data points that were available for comparison agree favorably with those reported here (Ataka & Asai, 1988; Howard *et al.*, 1988) except for Guilloteau, Riès-Kautt & Ducruix (1992) which

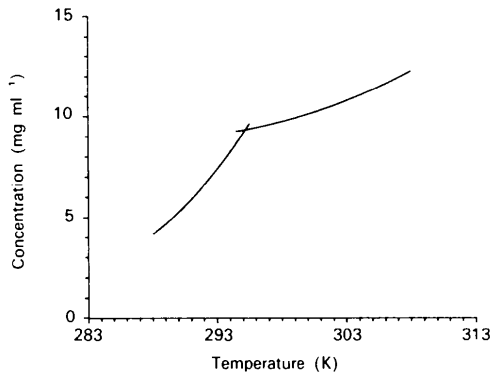

Fig. 3. Determination of the orthorhombic *versus* tetragonal equi-solubility point for pH 4.6, 3% NaCl. The phase transition occurs at 295 K. This figure also shows the difference in the slopes (response of solubility to temperature) of the two forms. Note that the low-temperature slope is definitely greater than the high-temperature slope.

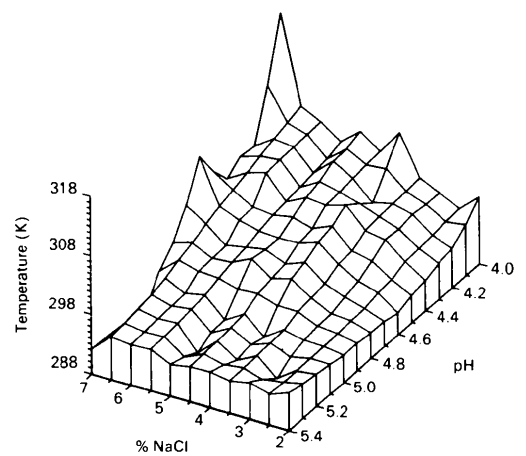

Fig. 4. Surface plot of intercept values for all salt concentrations and pH values. The equi-solubility points were a function of pH as well as precipitant concentration. The general tendencies of the points were to decrease with increasing pH. The precipitant concentration effects did not follow observable trends.

only had two data points in the same range. Their solubilities were two to three times higher but this difference is most likely due to the protein preparation using 0.05 *M* sodium acetate buffer. Howard *et al.* (1988) indicated that ionic strength (precipitant) was the dominant parameter influencing the solubility but also realized that temperature dependence was crucial. Hanging-drop crystallization techniques typically involve a simultaneous doubling of the protein and precipitant concentrations. Clearly, temperature and the precipitant ionic strength are important parameters influencing the solubility of proteins, but we have found that the buffer ionic strength also effects the solubility (work in progress).

The effects of pH on solubility have been exploited for the growth of protein crystals (McPherson, 1982). However, they are more generally applied in protein purification and only rarely systematically studied. With tetragonal lysozyme solubilities, pH effects were discussed briefly but no general trends were revealed. In fact, the pH effect on solubilities varied with the precipitant concentration (Ataka & Tanaka, 1986; Cole, Bryan & Bryan, 1969; Cacioppo & Pusey, 1991). Orthorhombic solubilities showed a more consistent pattern with solubilities decreasing with increasing pH at all salt concentations. A second pH effect was discovered when data from tetragonal and orthorhombic phase diagrams were combined to calculate the phase-change temperatures. A plot of the calculated equi-solubility points *versus* pH showed that the tetragonal→orthorhombic phase-change temperatures decreased with increasing pH for every salt concentation.

Most biochemical thermodynamic data have been obtained through the application of the van't Hoff equation,

$$\ln K_{eq} = \frac{-\Delta H^\circ}{R}\left(\frac{1}{T}\right) + \frac{\Delta S^\circ}{R},$$

where $K_{eq} = 1/C_{sat}$. C_{sat} is defined as the experimentally determined solubility of the protein. A plot of $\ln K_{eq}$ *versus* $1/T$, yields a straight line of slope $-\Delta H^\circ/R$ and intercept $\Delta S^\circ/R$, assuming that the change in the solute's heat capacity, ΔC_p, is zero. Values calculated for ΔH° and ΔS° for orthorhombic lysozyme ranged from -32.17 kJ mol^{-1} and -49.8 J K^{-1} mol (3% NaCl, pH 4.0) to -17.99 kJ mol^{-1} and 15.23 J K^{-1} mol (7% NaCl, pH 5.2), respectively. No trends involving these thermodynamic properties were observed, whereas both ΔH° and ΔS° were found to increase with increasing salt concentation and pH for tetragonal lysozyme (Cacioppo & Pusey, 1991). Ataka & Asai (1988) previously reported enthalpy values for the orthorhombic form to be -32 kJ mol^{-1} for 3 and 5% NaCl, which are comparable with those found in this study.

Another important empirical relationship introduced by Cohn (1925) has often been used for analysis of solubility data. The logarithm of the solubility is plotted *versus* the ionic strength, theoretically resulting in a straight line whose slope represents a salting-out constant while the ordinate intercept gives an extrapolated protein solubility at zero ionic strength. Following this approach and using the orthorhombic data for pH 4.6, 2–7% NaCl at 303 and 308 K, the plots obtained were concave instead of the expected straight line. This non-linearity has been noted previously with other crystal forms and precipitating ions for lysozyme (Ataka & Tanaka, 1986; Howard *et al.*, 1988; Riès-Kautt & Ducruix, 1989) and for crystals of concanavalin A (Mikol & Giegé, 1989). Bunn, Moews & Baumber (1971), using rennin, showed as expected that crystalline protein solubilities are lower than for an amorphous precipitate. Presumably, Cohn's work was performed on an amorphous precipitate. As we have previously pointed out, it may be that Cohn's relationship only holds for soluble→amorphous systems, and not soluble→crystalline (Cacioppo & Pusey, 1991).

Finally, we should like to highlight the importance of temperature in protein crystal growth research. In this case, it is the subtle shifts in protein structure which are thermally induced that are important. Given the dramatic shifts that have been observed in the catalytic properties of lysozyme over temperature changes of a few K, it is not surprising that there would be an accompanying change in the crystal form even though the net structural changes are very slight. It may be that some of the impurity effects recently found for tetragonal crystal growth are in fact due to the presence of orthorhombic lysozyme (Vekilov, Ataka & Katsura, 1993). This material may have been produced in the solutions by the use of varying temperatures to control nucleation and growth. Alternatively, it may have been in the initial protein as supplied by the vendor. Berthou & Jollès (1974) point out that the orthorhombic crystal form is particularly stable at tetragonal temperatures while the reverse is apparently not true. Orthorhombic lysozyme, with slightly different intermolecular interactions, would be a most pernicious impurity for the tetragonal crystal form.

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