

FOR THE RECORD

A structural basis for the interaction of urea with lysozyme

ASHLEY C.W. PIKE AND K. RAVI ACHARYA

School of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom

(RECEIVED September 21, 1993; ACCEPTED January 25, 1994)

Abstract: The effect of urea on the crystal structure of hen egg-white lysozyme has been investigated using X-ray crystallography. High resolution structures have been determined from crystals grown in the presence of 0, 0.7, 2, 3, 4, and 5 M urea and from crystals soaked in 9 M urea. All the forms are essentially isomorphous with the native type II crystals, and the derived structures exhibit excellent geometry and RMS differences from ideality in bond distances and angles. Comparison of the urea complex structures with the native enzyme (type II form, at 1.5 Å resolution) indicates that the effect of urea is minimal over the concentration range studied. The mean difference in backbone conformation between the native enzyme and its urea complexes varies from 0.18 to 0.49 Å. Conformational changes are limited to flexible surface loops (Thr 69–Asn 74, Ser 100–Asn 103), the active site loop (Asn 59–Cys 80), and the C-terminus (Cys 127–Leu 129). Urea molecules are bound to distinct sites on the surface of the protein. One molecule is bound to the active site cleft's C subsite, at all concentrations, in a fashion analogous to that of the *N*-acetyl substituent of substrate and inhibitor sugars normally bound to this site. Occupation of this subsite by urea alone does not appear to induce the conformational changes associated with inhibitor binding.

Keywords: crystal structure; lysozyme; urea denaturation

The interaction between denaturants and the 3-dimensional structure of proteins is of current interest, especially in terms of protein stability and folding. Knowledge of the events that take place at the onset of denaturation will provide important insights into the heterogeneous nature of protein stability and the relative susceptibilities of proteins to different types of denaturant. Many chemical agents are known to disrupt the folded conformation of a protein, but one of the most commonly used denaturants is urea.

Urea is a strong hydrogen bonding solvent that acts primarily by diminishing hydrophobic interactions in the close-packed interior of proteins (Tanford, 1968). Most studies concerning

the interactions and effects of urea on proteins have been carried out using spectroscopic methods. Until recently, this approach was sufficient to define the overall effects of urea on the conformation of the protein in solution but was unable to give detailed structural information about the precise nature of urea interaction with the entire molecule. Urea binds to hen egg-white lysozyme (HEWL) in solution (Warren & Gordon, 1970; Howarth & Lian, 1984; Makhatazde & Privalov, 1992). Nevertheless, HEWL is remarkably resistant to urea denaturation, and in the presence of up to 8 M urea, structural changes are slight and associated only with the nonhelical portions of the protein (Hamaguchi, 1958; Barnes et al., 1972).

Crystals of HEWL can be grown from solutions containing quite high concentrations of urea (Berthou & Jollès, 1973). Preliminary crystallographic analyses of such co-crystals and urea-soaked native crystals have demonstrated large intensity changes reflecting possible structural alterations within the protein (Berthou & Jollès, 1973; Snape, 1974; Snape et al., 1974). To investigate the structural basis of urea interaction with HEWL, we have determined high resolution crystal structures for HEWL over a wide range of urea concentrations and have compared these structures in an attempt to correlate the effect of increasing urea concentration with perturbations of the 3-dimensional structure of the enzyme.

Results

Increasing urea concentration (0.7–5 M) had little effect on the dimensions of the tetragonal unit cell, except in the case of the 9 M soaked crystals where the cell volume was reduced by about 8% (1.2 Å change in *a* and *b*, 1.9 Å change in *c*). However, increasing concentration did have a substantial effect on crystal size, as although it is possible to obtain crystals in up to 7 M urea, high resolution data could only be collected for the co-crystal complexes up to 5 M urea.

The refined structures are of high quality as assessed from the refinement parameters in spite of the presence of molar concentrations of urea (Table 1). Increasing urea concentration affects the flexibility of some of the surface side chains, promoting disorder-to-order transitions, most likely through the provision of additional hydrogen bonding partners in the urea complex

Reprint requests to: K.R. Acharya, School of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK; e-mail: k.r.acharya@midge.bath.ac.uk.

Table 1. Crystallographic data and refinement statistics

Condition	Maximum resolution (Å)	Number of unique reflections	% Completeness of data ^a	R_{symm}^b	Number of water molecules	Number of urea molecules	Crystallographic R -factor ^c (8 Å-max ^a) ($F \geq 2\sigma(F)$)	RMSD of bonds (Å)
Native HEWL	1.49	15,803	93	11.90	112	0	18.7	0.013
HEWL + 0.7 M urea	1.50	14,881	92	9.14	111	3	19.2	0.014
HEWL + 2.0 M urea	1.62	12,922	92	8.68	94	3	19.8	0.015
HEWL + 3.0 M urea	1.61	11,534	91	9.39	104	3	18.4	0.015
HEWL + 4.0 M urea	1.75	9,038	84	9.24	94	8	17.8	0.015
HEWL + 5.0 M urea	1.92	7,527	89	15.04	108	8	17.2	0.016
HEWL + 9.0 M urea	1.56	13,466	93	8.39	86	9	19.3	0.015

^a Up to maximum resolution quoted.

$${}^b R_{\text{symm}} = \frac{\sum_h \sum_{l=1}^n |\bar{I}_h - I_{hl}|}{\sum_h \bar{I}_h}$$

$${}^c R_{\text{cryst}} = \frac{\sum_h |F_{h, \text{obs}} - F_{h, \text{calc}}|}{\sum_h F_{h, \text{obs}}}$$

structures. This "ordering" is accompanied by a reduction in the side chain's thermal parameters (B -factors) compared to the native structure. The way urea acts to reduce the overall surface mobility of the protein is reflected in the average B -factor value for the enzyme, which is significantly lower in the urea complexes. The most noticeable stabilization occurs at surface loops that are poorly ordered in the native structure. Urea does not have any disordering or destabilizing effect on either the overall or local regions of the crystal structure of HEWL even at high concentrations. Changes in the accessibilities of disulfide bonds, affected at high urea concentrations in solution, are negligible (Barnes et al., 1972). Reassuringly, there is no evidence of covalent modification of the protein—in particular the carbamylation of free amino or sulfhydryl groups, which would be seen if the urea solutions used were "contaminated" with cyanate (Stark et al., 1960).

The presence of urea molecules that can be clearly defined in the electron density maps is dependent on urea concentration. In the 0.7 M structure only 3 molecules are seen compared to

a total of 9 in the 9 M complex. The first molecule (urea 250) lies on the dyad axis between the C-terminal carboxyl and its symmetry-related partner. It is positioned so that the carbonyl oxygen lies along the axis, making hydrogen bonds to the ends of Arg 14 and Arg 14# (symmetry-related molecule)—one of the NH_2 groups hydrogen bonds to the main-chain CO of Ala 10 and the C-terminal oxygen of Leu 129, whereas the other makes identical contacts in the symmetry-related molecule (Fig. 1A). The second urea molecule (urea 251) also lies on a dyad axis with the carbonyl group of the urea orientated along the axis, hydrogen bonding to the $\text{O}^\gamma 1$ of Thr 43 and its symmetry-related partner (Fig. 1B). The final urea molecule (urea 252) lies in HEWL's active site cleft—more precisely in subsite C, the "acetamido-specific" binding pocket, where it mimics the interactions of the N -acetyl group present on both substrates and inhibitors of lysozyme (Blake et al., 1967a; Strynadka & James, 1991). The molecule is bound between the main-chain CO of Ala 107 and the main-chain NH of Asn 59. In addition, it hydrogen bonds via an NH_2 group to the N^ϵ group of Trp 108

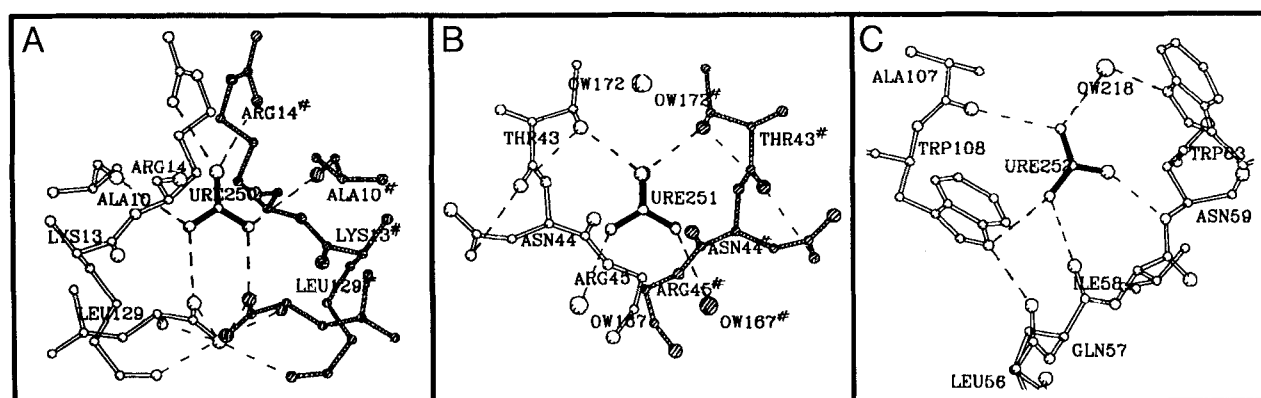


Fig. 1. Environments of the 3 urea molecules present in all the urea complexes. Only the relevant interactions are shown. The urea molecules are drawn with black bonds and the symmetry-related molecules are shaded. The dashed lines represent hydrogen bonds. **A:** Urea 250. **B:** Urea 251. **C:** Urea 252—subsite C urea.

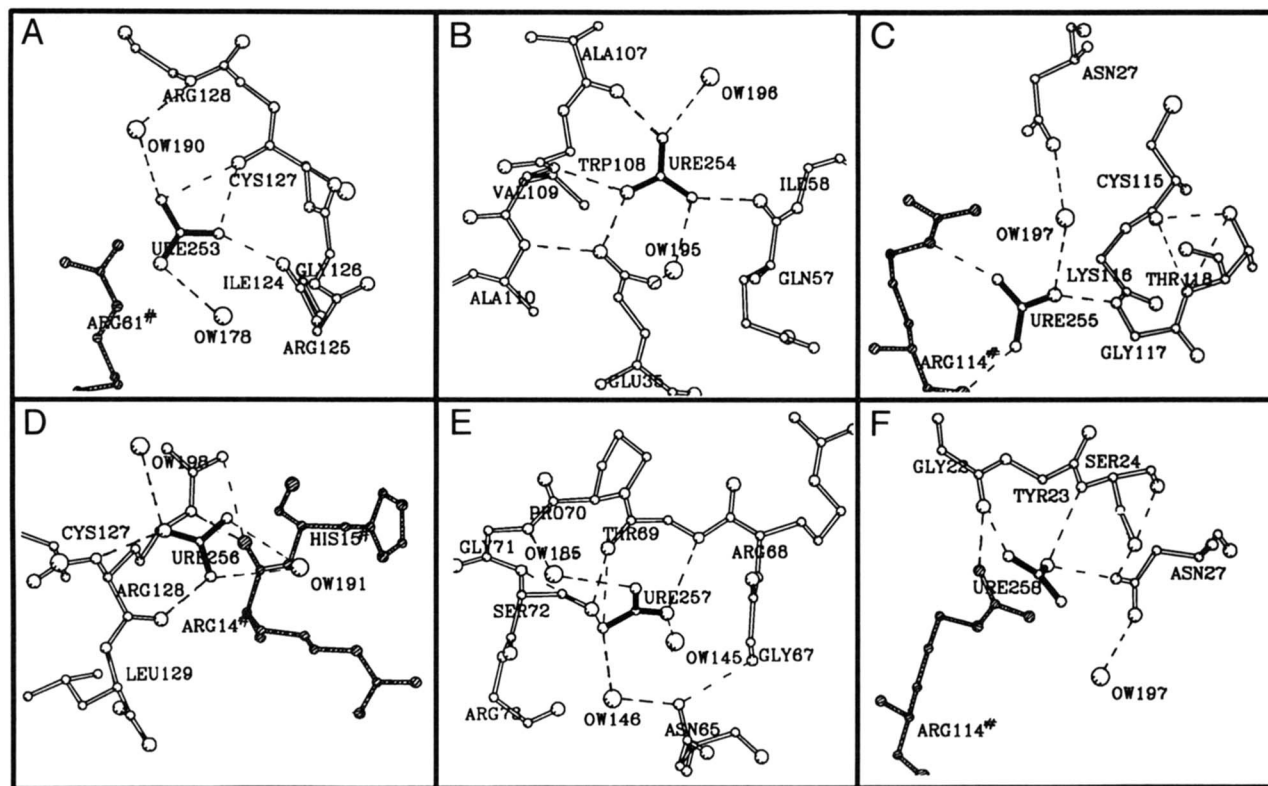


Fig. 2. Environments of the urea molecules present at concentrations greater than 4 M. Only the relevant interactions are shown. The urea molecules are drawn with black bonds and the symmetry-related molecules are shaded. The dashed lines represent hydrogen bonds. **A:** Urea 253. **B:** Urea 254. **C:** Urea 255. **D:** Urea 256. **E:** Urea 257. **F:** Urea 258.

and to the main-chain CO of Gln 57, as well as to a water molecule that is bound to the N^ε1 group of Trp 63 (Fig. 1C).

In both the 2 M and 3 M complexes no additional urea molecules can be clearly defined although ureas 250–252 are less mobile than in the 0.7 M structure. Five additional urea molecules can be assigned to peaks in the electron density of the 4 M structure. Three of these are located at or near the C-terminus (urea 253, 255, 256), one in the active site cleft (urea 254), and one near Ser 24 (urea 258). These urea molecules interact with the enzyme via multiple hydrogen bonds made primarily to the peptide groups but also with certain side chains and water molecules (Fig. 2). In the 5 M structure, all these urea molecules are present, apart from the one bound to the NH of Gly 117 (urea 255). Finally, the 9 M soaked structure possesses all the urea molecules seen in the 4 M map, but, in addition, a molecule (urea 257) can be clearly assigned to the Pro 70 loop region on the 2-fold axis (Fig. 2E). The relative positions of the 9 molecules in the 9 M urea-soaked structure are shown in Figure 3.

A global comparison of the structures shows that they are very similar and the RMSD differences (RMSD) between them are small (Table 2). When the urea complex structures (0.7–5 M) are compared with the native structure, the major differences in backbone conformation occur around the crystal contact loop regions Thr 69–Asn 74 (2-fold axis) and Ser 100–Asn 103, where the structure is flexible and poorly defined. As mentioned above, these loop conformations are stabilized in the presence of urea. More significant however, is a shift that occurs at the C-terminus at urea concentrations greater than 0.7 M. In the 2–9 M struc-

tures, the C-terminal dipeptide rotates toward the N-terminal helix and the body of the enzyme with an axial displacement for the C-terminal carboxylate of about 2.0 Å. This shift allows the rotated C-terminal dipeptide to be stabilized through favorable interactions with the urea molecule (urea 250) located on the dyad axis near Ala 10 (see Fig. 1A).

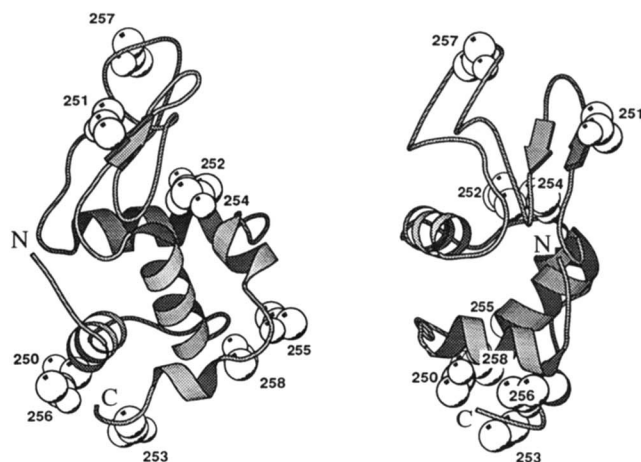


Fig. 3. Location of the 9 urea molecules in the 9 M urea-soaked complex. The diagram was produced using the program MOLSCRIPT (Kraulis, 1991).

Table 2. RMSD between the native and urea complex structures

Structure	RMSD (Å)	
	Main chain only	All atoms
+0.7 M urea	0.18	0.51
+2.0 M urea	0.21	0.85
+3.0 M urea	0.23	0.67
+4.0 M urea	0.24	0.66
+5.0 M urea	0.31	0.62
+9.0 M urea	0.49	0.86

The 9 M urea-soaked structure exhibits slightly more main-chain differences. The majority of these are due to a "compacting" of the structure in response to the 8% reduction in unit cell volume that occurs at this high urea concentration. The only major backbone difference occurs between Asn 59 and Cys 80, where the deviation from the native structure varies from 0.6 Å to as much as 1.0 Å. The most dramatic change in this loop is a peptide flip that occurs between Arg 73 and Asn 74, a region that molecular dynamics simulations have shown to be highly flexible (Post et al., 1986). Although the appearance of the urea molecule (urea 257) bound near Pro 70 accompanies this main-chain shift, it is unclear whether it binds after the conformational change has taken place or whether the shift occurs as a direct consequence of the urea molecule's presence. When the side chains are compared, the orientation of Trp 62 seems to be the most sensitive to urea concentration. The ring atoms of Trp 62 are gradually shifted in response to increasing urea concentration so that, in the 9 M complex, the mean shift of the ring atoms is about 1 Å. Although this movement is similar to one that occurs on inhibitor binding, urea does not elicit the global changes in conformation associated with substrate binding. These observations contrast the effects of urea in solution (Lumb & Dobson, 1992). Urea seems to have a minimal effect on the bound waters in the co-crystal structures (0.7–5 M) and only reduces the number seen in the 9 M urea-soaked structure slightly (see Table 1). Although the overall water structure of each complex appears to be unique, the positions of certain buried water molecules are conserved even at high concentrations of urea. The positions of these molecules are also highly conserved in the structures of *c*-type lysozymes from other species (Blake et al., 1983).

Discussion

The detailed crystallographic studies of lysozyme–urea complexes show that contrary to previous suggestions (Snape et al., 1974), no significant conformational changes occur at high urea concentrations. This apparent conformational stability is not surprising when one considers HEWL's resistance to urea denaturation (Hamaguchi, 1958). However, it remains possible that in the crystallization process we are inadvertently favoring the "native" conformational state because to grow sizeable co-crystals at high urea concentrations, it is necessary to increase the protein concentration and lower the crystallization temperature (Berthou & Jollès, 1973).

Even though the enzyme shows no signs of denaturation, we have been able to describe the urea binding sites on the protein in some detail and observe subtle conformational changes that take place within the crystal structure on exposure to urea. All these changes are at regions on the surface of the molecule that are known to be relatively flexible. Surprisingly, the effect of increasing urea concentration is to stabilize these flexible parts of the molecule and to allow a relaxation of the crystal-packing contacts. Because the dynamics of the surface residues and loops are greatly influenced by solvent, it appears that the majority of perturbations seen here result from urea's disruption of the solvent structure.

Recent calorimetric studies suggest that urea interacts with polar residues of proteins in both the folded and unfolded states (Makhatadze & Privalov, 1992). Here, urea interacts with the enzyme via multiple hydrogen bonds made primarily to peptide groups and polar side chains. In contrast to results from a similar crystallographic study of denaturant binding to α -chymotrypsin (Hibbard & Tulinsky, 1978), none of the urea binding sites on HEWL are particularly hydrophobic in nature. Further detailed experimental studies on proteins less resistant to denaturation are needed to get a better insight into the molecular mechanisms of urea's action.

Experimental procedures

Crystals of the HEWL–urea complexes were grown by batch and hanging drop methods (Berthou & Jollès, 1973; Wilson et al., 1991). Co-crystallization trials were carried out at room temperature, 13 °C and 4 °C in the presence of urea (0.5–7 M). The 9 M urea soaking studies were performed using native crystals as described by Snape et al. (1974). A commercial sample of HEWL was used throughout (HEWL 3 \times crystallized from Sigma), and all urea solutions were freshly prepared from analytical grade urea (Fisons). X-ray diffraction data were collected from the native and urea-complex crystals using a Siemens Area Detector with CuK α X-rays generated by a rotating anode and collimated by a graphite monochromator. All the crystals belonged to the tetragonal space group P4 $_2$ 2 $_1$ 2 with 1 molecule per asymmetric unit. Data were processed with the XENGEN package of programs to give a scaled set of intensities (Howard et al., 1987) (Table 1).

The atomic co-ordinates of the tetragonal type II form of HEWL (Blake et al., 1967b), recently refined by Acharya (unpubl. results), were used as a starting point for the refinement of our native structural data. Refinement of the HEWL–urea complex data was carried out using XPLOR (Brünger, 1990) with the refined native co-ordinates as a starting model. In the final stages, after the *R*-factors had converged, bound water molecules were included and urea molecules were modeled into appropriate peaks in the electron density maps using the structural parameters determined by Caron and Donohue (1963) (Table 1).

The native and urea-complex coordinates will be deposited in the Brookhaven Protein Data Bank.

Acknowledgments

We thank Sir David Phillips for providing the native HEWL coordinates and Professor Anthony Rees for his encouragement. A.C.W.P is supported by the Wellcome Trust.

References

- Barnes KP, Warren JR, Gordon JA. 1972. Effect of urea on the circular dichroism of lysozyme. *J Biol Chem* 247:1708-1712.
- Berthou J, Jollès P. 1973. The influence of urea on crystallization and polymorphism of hen lysozyme. *FEBS Lett* 31:189-192.
- Blake CCF, Johnson LN, Mair GA, North ACT, Phillips DC, Sarma VR. 1967a. Crystallographic studies of the activity of hen egg-white lysozyme. *Proc R Soc Lond B* 167:378-388.
- Blake CCF, Mair G, North ACT, Phillips DC, Sarma VR. 1967b. On the conformation of the hen egg-white lysozyme molecule. *Proc R Soc Lond B* 167:365-377.
- Blake CCF, Pulford W, Artymiuk PJ. 1983. X-ray studies of water in crystals of lysozyme. *J Mol Biol* 167:693-723.
- Brünger A. 1990. *XPLOR manual—Version 2.1: A system for crystallography and NMR*. New Haven, Connecticut: Yale University.
- Caron A, Donohue J. 1964. Three-dimensional refinement of urea. *Acta Crystallogr* 17:544-546.
- Hamaguchi K. 1958. Studies on the denaturation of lysozyme. II. Urea denaturation. *J Biochem (Tokyo)* 45:79-88.
- Hibbard LS, Tulinsky A. 1978. Expression of functionality of α -chymotrypsin. Effects of guanidine hydrochloride and urea in the onset of denaturation. *Biochemistry* 17:5460-5468.
- Howard A, Gilliland G, Finzel B, Poulos T. 1987. The use of an imaging proportional counter in macromolecular crystallography. *J Appl Crystallogr* 20:383-387.
- Howarth OW, Lian LY. 1984. Hen egg white lysozyme: Carbon-13 nuclear magnetic resonance assignments and dependence of conformational flexibility on inhibitor binding and temperature. *Biochemistry* 23:3522-3526.
- Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* 24:946-950.
- Lumb KJ, Dobson CM. 1992. ^1H nuclear magnetic resonance studies of the interaction of urea with hen lysozyme. *J Mol Biol* 227:9-14.
- Makhataдзе GI, Privalov PL. 1992. Protein interactions with urea and guanidinium chloride. *J Mol Biol* 226:491-505.
- Post CB, Brooks BR, Karplus M, Dobson CM, Artymiuk PJ, Cheatham JC, Phillips DC. 1986. Molecular dynamics simulations of native and substrate-bound lysozyme. *J Mol Biol* 190:455-479.
- Snape KW. 1974. A crystallographic study of the interaction of urea with lysozyme [thesis]. Oxford, UK: Oxford University.
- Snape KW, Tjian R, Blake CCF, Koshland DE. 1974. Crystallographic study of the interaction of urea with lysozyme. *Nature* 250:295-298.
- Stark GR, Stein WH, Moore S. 1960. Reactions of the cyanate present in aqueous urea with amino acids and proteins. *J Biol Chem* 235:3177-3181.
- Strynadka NCJ, James MNG. 1991. Lysozyme revisited: Crystallographic evidence for distortion of an *N*-acetylmuramic acid residue bound in site D. *J Mol Biol* 220:401-424.
- Tanford C. 1968. Protein denaturation. *Adv Protein Chem* 23:121-275.
- Warren JR, Gordon JA. 1970. Denaturation of globular proteins. II. The interaction of urea with lysozyme. *J Biol Chem* 247:1708-1712.
- Wilson LJ, Bray TL, Suddath FL. 1991. Crystallization of proteins by dynamic control of evaporation. *J Crystal Growth* 110:142-147.