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Water-Mediated Transformations in Protein Crystals

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Abstract

Different crystal forms of bovine pancreatic ribonuclease A and hen egg white lysozyme, 2Zn insulin, 4Zn insulin and crystals of concanavalin A were examined under controlled environmental humidity in the relative humidity (r.h.) range of 100 to 75%. Many of them, but not all, undergo reversible structural transformations as evidenced by discontinuous changes in the diffraction pattern, the unit-cell dimensions and the solvent content. Tetragonal, orthorhombic and monoclinic lysozyme and a new crystal form of ribonuclease A show transformations at r.h.'s above 90%. Monoclinic lysozyme transforms at low r.h. to another monoclinic form with nearly half the original cell volume. The well known monoclinic form of ribonuclease A grown from aqueous ethanol solution undergoes two transformations while the same form grown from 2-methyl-2,4-pentanediol (MPD) solution in phosphate buffer does not transform at all. Soaking experiments involving alcohol solutions demonstrate that MPD has the effect of decreasing the r.h. at which the transformation occurs. Triclinic lysozyme, 2Zn insulin, 4Zn insulin and the crystals of cancanavalin A do not transform in the 100 to 75% r.h. range before losing crystallinity. The results obtained so far indicate that the crystal structure has a definite influence on water-mediated transformations. The transformations do not appear to depend critically on the amount of solvent in the crystals but the r.h. at which they occur is influenced by the composition of the solvent. The transformations appear to involve changes in crystal packing as well as conformational transitions in protein molecules. The present investigations and other related studies suggest that water-mediated transformations in protein crystals could be very useful in exploring conformational transitions in and the hydration of proteins.

Introduction

The role of water in biological systems can hardly be overemphasized. Proteins, like other biomolecules, almost invariably exist and function in an aqueous environment. Considerable progress has recently been made through X-ray studies in the understanding of the structure of water surrounding protein molecules (Blake, Pulford & Artymiuk, 1983; Finney, 1979; Watenpaugh, Sieker & Jensen, 1979; Sakabe, Sakabe & Sasaki, 1980; Teeter, 1984). The hydration of proteins and the effects of protein-water interactions have been studied in considerable detail by other techniques as well. Such studies had earlier indicated the dependence of protein conformation on the amount of water present in the sample (Kuntz & Kauzmann, 1974). Subsequent physicochemical and biochemical studies on lysozyme led to a hydration model that did not involve significant changes in protein conformation (Careri, Gratton, Yang & Rupley, 1980). More recently, there has been a spurt in the investigations on protein-water interactions and their consequences (Poole & Finney, 1983, 1984; Baker, Hansen, Bhaskara Rao & Bryan, 1983). Many of these investigations clearly show that a change in hydration is often accompanied by conformational changes. They also re-emphasize the importance of water in protein action.

The first and, as far as we are aware, only systematic attempt to study the effects of the amount and the composition of the aqueous solution surrounding protein molecules on crystal structure were made in the late nineteen forties and early fifties on haemoglobin (Boyes-Watson, Davidson & Perutz, 1947;

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Huxley & Kendrew, 1953). The results were then interpreted in terms of the movement of layers of protein molecules. The other ramifications of the results were not then further explored. Our interest in this problem was generated by the observations, one accidental and the other planned, of water-mediated transformations in a new crystal form of ribonuclease A and tetragonal lysozyme (Salunke, Veerapandian & Viyayan, 1984). Such transformations appeared to provide a useful handle for exploring possible water-induced conformational transitions, as well as the general problem of protein hydration. A systematic search for water-mediated transformations in different crystal forms of four well known proteins, namely bovine pancreatic ribonuclease A, hen egg white lysozyme, porcine insulin and concanavalin A, was therefore undertaken. The results of this search are discussed here.

Experimental

The proteins used in these investigations were obtained commercially from Sigma Chemical Company, USA. The new crystal form of ribonuclease A (Salunke, Veerapandian & Vijayan, 1984), the well known monoclinic form of ribonuclease A grown from aqueous ethanol as well as from MPD solution in phosphate buffer (Kartha, Bello & Harker, 1967; Carlisle, Palmer, Mazumdar, Gorinsky & Yeates, 1974), tetragonal lysozyme (Blake, Koenig, Mair, North, Phillips & Sarma, 1965), orthorhombic lysozyme (Artymiuk, Blake, Rice & Wilson, 1982), monoclinic lysozyme (Hogle, Rao, Mallikarjunan, Beddell, McMullan & Sundaralingam, 1981) and triclinic lysozyme (Moult, Yonath, Traub, Smilansky, Podjarny, Rabinovich & Saya, 1976), 2Zn insulin (Adams, Blundell, Dodson, Dodson, Viyayan, Baker, Harding, Hodgkin, Rimmer & Sheat, 1969), 4Zn insulin (Bentley, Dodson, Dodson, Hodgkin & Mercola, 1976) and concanavalin A (Hardman, Wood, Schifer, Edmundson & Ainsworth, 1971) were prepared using methods described in the literature. The relative humidity (r.h.) of the atmosphere surrounding the crystals was maintained at desired values by introducing appropriate saturated salt solutions into the glass capillaries containing the crystals (Rockland, 1960). Diffraction patterns were then recorded at r.h. 98, 95, 93, 90, 88, 84, 79.5, 75 and 66%. The r.h. in the capillary was taken to be 100% when distilled water was used instead of salt solutions. Typically, crystals were allowed to equilibrate for 24 h after the salt solution was introduced, before X-ray photographs were taken. When crystals grown from one type of solution were transferred to another type [*e.g.* when the new crystal form of ribonuclease A grown from acetone solution in tris buffer (2-amino-2-hydroxymethyl-1,3-propanediol) was transferred to MPD solution in phosphate buffer], they were soaked in the new solution for about 10 d before further experiments were conducted on the crystals. All the crystals were characterized using 15° zonal precession photographs. The solvent content of the crystals was determined using Matthew's (1968) method assuming the partial specific volume, $\bar{v}$, to be 0·74 in all cases.

Table 1. *Summary of results of experiments involving variation of relative humidity*

Pancreatic ribonuclease A	
TACE:	Transforms between r.h. 95 and 93%
	100% r.h.: $P2_1$; $a = 33{\cdot}8$, $b = 106{\cdot}4$, $c = 31{\cdot}8$ Å, $\beta = 98{\cdot}3°$; $Z = 4$; solvent content = 40%
	93% r.h.: $P2_1$; $a = 33{\cdot}5$, $b = 105{\cdot}9$, $c = 30{\cdot}6$ Å; $\beta = 101{\cdot}9°$; $Z = 4$; solvent content = 36%
WETL:	Transforms between r.h. 93 and 90% and again between 88 and 84%
	100% r.h.: $P2_1$; $a = 30{\cdot}3$, $b = 38{\cdot}6$, $c = 53{\cdot}3$ Å, $\beta = 106{\cdot}0°$; $Z = 2$; solvent content = 44%
	90% r.h.: $P2_1$; $a = 29{\cdot}7$, $b = 38{\cdot}4$, $c = 52{\cdot}3$ Å, $\beta = 112{\cdot}8°$; $Z = 2$; solvent content = 39%
	84% r.h.: $P2_1$; $a = 30.4$, $b = 33{\cdot}3$, $c = 52{\cdot}8$ Å, $\beta = 113{\cdot}5°$; $Z = 2$; solvent content = 31%
PMPD:	Does not transform in the r.h. range 100 to 75%
HEW lysozyme	
Tetragonal:	Transforms between r.h. 93 and 90%
	100% r.h.: $P4_32_12$; $a = 79{\cdot}6$, $c = 38{\cdot}3$ Å; $Z = 8$; solvent content = 41%
	90% r.h.: $P4_32_12$; $a = 78{\cdot}4$, $c = 37{\cdot}3$ Å; $Z = 8$; solvent content = 37%
Orthorhombic:	Transforms between r.h. 93 and 90%
	100% r.h.: $P2_12_12_1$; $a = 59{\cdot}5$, $b = 68{\cdot}6$, $c = 30{\cdot}7$ Å; $Z = 4$; solvent content = 43%
	90% r.h.: $P2_12_12_1$: $a = 55{\cdot}1$, $b = 68{\cdot}2$, $c = 30{\cdot}6$ Å; $Z = 4$; solvent content = 38%
Monoclinic:	Transforms between r.h. 93 and 90%
	100% r.h.: $P2_1$; $a = 28{\cdot}3$, $b = 63{\cdot}1$, $c = 60{\cdot}4$ Å, $\beta = 90{\cdot}5°$; $Z = 4$; solvent content = 34%
	90% r.h.: $P2_1$; $a = 27{\cdot}0$, $b = 59{\cdot}1$, $c = 31{\cdot}5$ Å, $\beta = 112{\cdot}3°$; $Z = 2$; solvent content = 23%
Triclinic:	Does not transform in the r.h. range of 100 to 75%.

2Zn insulin, 4Zn insulin and the crystals of concanavalin A do not transform in the r.h. range 100 to 75%.

Results

The main results of the experiments outlined above are summarized in Table 1. In this table and in the rest of the paper, the new crystal form of ribonuclease A grown from acetone solution in tris buffer, the well known form grown from aqueous ethanol and the same form grown from MDP solution in phosphate buffer are denoted as TACE, WETL and PMPD respectively. The names of the different crystal forms of the other proteins used in this paper are self-explanatory.

As can be seen from Table 1, many of the crystals examined in the present study exhibit transformations

when the r.h. of the environment is reduced. The transformation is reversible wherever it occurs, and it is clearly distinguishable from the normal drying process. The quality of the diffraction pattern remains nearly the same before and after the transformation. The drying process is, however, accompanied by the progressive deterioration of the quality of the pattern. The diffraction patterns were not monitored at close intervals below r.h. 75%. Many crystal forms lose or begin to lose their crystallinity when the r.h. is reduced to 66%, and the work reported here has primarily monitored structural changes in the r.h. range of 100 to 75%.

Ribonuclease A

TACE transforms between 95 and 93% r.h. WETL undergoes two transformations, one between 93 and 90%, and the other between 88 and 84% r.h. These transformations are accompanied by abrupt changes in the unit-cell volume (Fig. 1) and the diffraction pattern (Fig. 2). These changes are not achieved through isotropic changes in cell dimensions, because the axial lengths decrease by different extents, coupled with a change in the monoclinic angle, indicating the occurrence of molecular rearrangements within the crystal. The quality of the diffraction pattern remains good and the unit-cell volume remains nearly constant until the r.h. is reduced to 88% in the case of TACE and below 75% in the case of WETL. Further reduction in humidity results in a continuous reduction in cell volume accompanied by a progressive deterioration of the quality of the pattern in the case of TACE. WETL loses its crystallinity at 66% r.h., the only point below 75% at which the diffraction pattern was recorded.

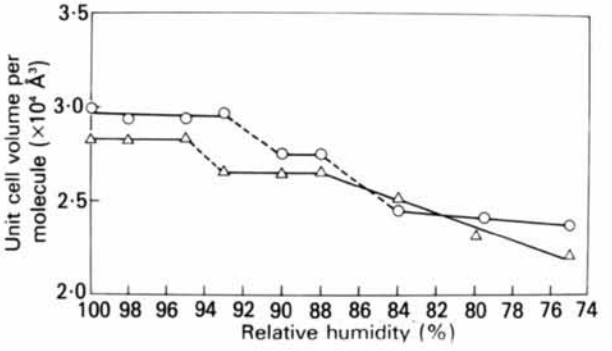


Fig. 1. Variation of unit-cell volume per molecule as a function of relative humidity in the crystal forms TACE (△) and WETL (○) of ribonuclease A.

Lysozyme

All but one of the four crystal forms of HEW lysozyme, examined in the present study, undergo water-mediated transformations. The variation in the cell volumes of these forms as a function of humidity is shown in Fig. 3. The transformations are accompanied, as in the case of the two forms of the ribonuclease A described earlier, by substantial changes in intensities (see, for example, Fig. 4). However, the changes in the diffraction patterns and the cell volumes of the lysozyme crystals appear to be somewhat less abrupt than those in the two forms of ribonuclease A. Thus, the tetragonal, the orthorhombic and the monoclinic forms clearly transform between 93 and 90% r.h. However, the cell volumes in each case exhibit continuous, though small, variations before and after the transformations. In parallel, small changes occur in the intensities as well, but they are much less marked than the changes that accompany the transformations.

The four crystal forms of HEW lysozyme do not behave in the same way when the humidity of the environment is systematically reduced. The tetragonal and the orthorhombic forms, which have comparable solvent content, behave in a similar manner. Both of them transform between 93 and 90% r.h. and the diffraction pattern in both cases disappears at 84%

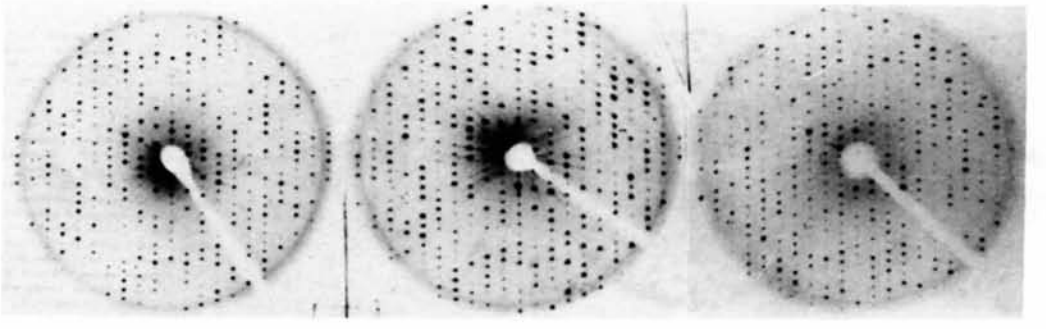


Fig. 2. *h*0*l* 15° precession photographs from crystal form WETL of ribonuclease A at (*a*) 100%, (*b*) 90% and (*c*) 84% relative humidity.

r.h. The monoclinic form with a low solvent content of 33.5% also transforms between 93 and 90% r.h., but the quality of the diffraction pattern from the low-humidity form remains good even at a r.h. as low as 75%. The triclinic form, which is crystallized in the same way as the monoclinic form and has comparable solvent content, however does not transform even at 75% r.h.

Among the transformations in the different forms of HEW lysozyme, that of the monoclinic form is the most remarkable, in that the cell volume is reduced to less than half during the transformation. It may be recalled that monoclinic lysozyme ($M1$) with cell dimensions $a = 28{\cdot}3$, $b = 63{\cdot}1$, $c = 60{\cdot}4$ Å and $\beta = 90{\cdot}5°$ has two molecules in the asymmetric unit (Hogle, Rao, Mallikarjunan, Beddell, McMullan & Sundaralingam, 1981). The molecular arrangement is such that the unit cell is pseudo B centred. The two crystallographically non-equivalent molecules become crystallographically equivalent in the low r.h. form making the cell truly B centred. The B-centred cell can now be transformed, as shown in Fig. 5, into a primitive monoclinic cell with half the cell volume, with identical b^* and c^*. The $0kl$ precession photographs from the two forms are given in Fig. 6 for comparison.

Insulin and concanavalin A

No transformation was observed in 2Zn insulin in the r.h. range 100 to 93%. The diffraction pattern loses its quality substantially at 90% r.h., and almost disappears when the humidity is further reduced. 4Zn insulin, however, retains the quality of the pattern even at a r.h. as low as 75%, but without any evidence for water-mediated transformation. The pattern almost disappears at r.h. 66%. The crystals of concanavalin A exhibit a behaviour similar to that of 4Zn insulin.

Effect of MPD on transformation

As noted earlier, the crystal form of ribonuclease A grown from aqueous ethanol (WETL) transforms between 93 and 90% r.h. The same form obtained from MPD solution in phosphate buffer (PMPD), however, does not transform even when the r.h. is

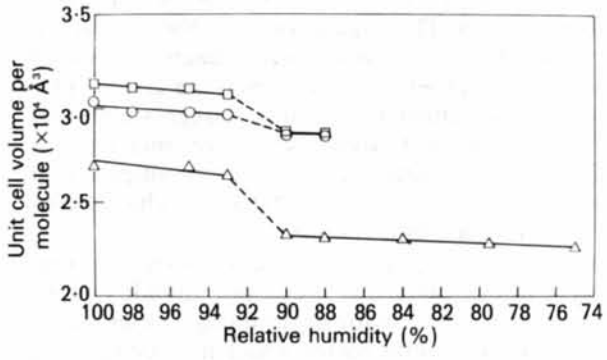


Fig. 3. Variation of unit-cell volume per molecule as a function of relative humidity in tetragonal (○), orthorhombic (□) and monoclinic (△) lysozyme.

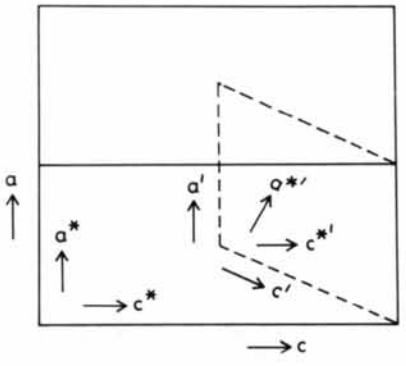
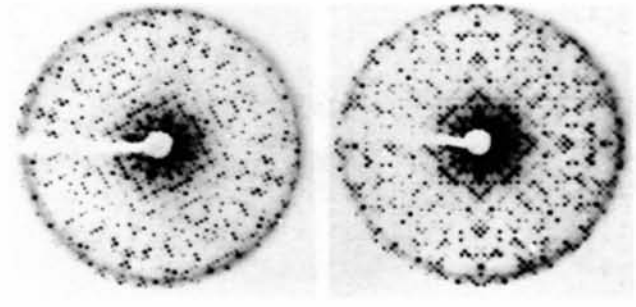
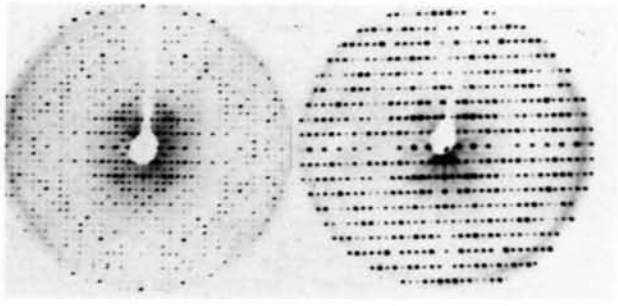


Fig. 5. The relationship between the unit cells of the high- and low-humidity forms of monoclinic lysozyme. The primed symbols correspond to the low-humidity form.



(*a*) (*b*)

Fig. 4. *hk*0 15° precession photographs from tetragonal lysozyme at (*a*) 100% and (*b*) 90% relative humidity.



(*a*) (*b*)

Fig. 6. $0kl$ 15° precession photographs from monoclinic lysozyme at (*a*) 100% and (*b*) 90% relative humidity.

reduced to 75%. The cell volume also remains unchanged at the reduced r.h. When WETL was soaked in MPD solution in phosphate buffer, the soaked crystals behaved exactly like PMPD with respect to variation in humidity. Likewise, PMPD soaked in aqueous ethanol behaves exactly like WETL. TACE transforms between 95 and 93% r.h. These crystals when soaked in MPD solution in phosphate buffer transform between 88 and 84% r.h. The same result is obtained when they are soaked in aqueous MPD solution.

No crystal form of HEW lysozyme obtained from solutions containing organic solvents is known. However, in view of the above results, the tetragonal crystals of lysozyme soaked in a 1:1 mixture of their mother liquor and MPD were examined for water-mediated transformations. Although the original crystals transformed between 93 and 90% r.h., the crystals soaked in this solution transformed only between r.h. 84 and 79.5%.

Discussion

Reversible water-mediated transformations appear to be a widespread, though not universal (at least in the 100-75% r.h. range), phenomenon among protein crystals. The *amount* of solvent in a given crystal does not appear to be critical in determining whether it transforms or not. For example, the volume of the solvent is the same in forms WETL and PMPD of ribonuclease A. One of them transforms while the other does not. On the other hand, the crystal structure has a definite influence on water-mediated transformation. For instance, although monoclinic lysozyme and triclinic lysozyme are obtained from the same mother liquor and have comparable solvent content, the former transforms between 93 and 90% r.h., whereas the latter does not transform even when the r.h. is reduced to 75%.

The effect of MPD on the r.h. at which the transformation occurs emphasizes the importance of co-solvents in water-mediated transformations. MPD stabilizes the high-humidity form; in the presence of MPD, the r.h. of transformation is reduced substantially. This could possibly be due to the higher boiling point of MPD; the bulk water that evaporates might be preferentially replaced by MPD. Preferential hydration of protein molecules in the presence of MPD, suggested by earlier solution studies on ribonuclease A (Pittz & Bello, 1971; Pittz & Timasheff, 1978) also cannot be ruled out. The co-solvent, however, does not appear to have any significant influence on the crystal structure before or after transformation. The soaking of crystals in MPD does not lead to any change in the diffraction pattern. More importantly, the diffraction patterns of the transformed crystals remain the same irrespective of the presence or the absence of MPD in the mother liquor, although the presence of MPD leads to a substantial reduction in the r.h. at which the transformation occurs.

A comprehensive elucidation of the structural changes that accompany water-mediated transformations must obviously await the complete X-ray analysis of the low-humidity forms of protein crystals. *A priori* the transformation may be accompanied by changes in the packing of molecules in the crystal, conformational changes in the protein molecule, or a combination of the two. Obviously, it is the possible conformational changes that are of particular interest. Although the available X-ray evidence is insufficient to prove that they do occur, recent studies on the flexibility of protein molecules (Frauenfelder, Petsko & Tsernoglou, 1979; Artymiuk, Blake, Grace, Oatley, Phillips & Sternberg, 1979; Huber, 1979; Wagner & Wuthrich, 1982; Wagner, 1983; Ribeiro, King & Jardetzky, 1983; Vijayan & Salunke, 1984), including that caused by hydration (Baker, Hansen, Bhaskara Rao & Bryan, 1983; Poole & Finney, 1984), suggest that conformational changes may occur in structural transformations caused by loss of water. In addition to changes in humidity, changes in ionic strength or the pH of the medium are also known to cause structural transformations in protein crystals. Extensive conformational changes are known to occur in the transformation of 2Zn to 4Zn insulin caused by a change in ionic strength (Bentley, Dodson & Lewitova, 1978). Similarly, the molecular structure of adenylate kinase changes substantially during the transformation of its crystals mediated by a change in pH (Sachsenheimer & Schulz, 1977). The changes in the unit-cell dimensions and the diffraction pattern during water-mediated transformations are comparable to those observed in these transformations. This again points to the likelihood of conformational changes being caused by the reduction of the humidity of the environment. In any case, the changes in the unit-cell dimensions themselves indicate conformational changes in one of the crystal forms examined in the present study. The high-humidity form of monoclinic lysozyme contains two molecules in the asymmetric unit with different conformations (Rao, Hogle & Sundaralingam, 1983). The two molecules become crystallographically equivalent in the low-humidity form. This can happen only if conformational changes occur at least in one of the molecules.

It has been shown that water molecules in protein crystals can be classified into ordered or bound water and disordered or bulk water (Blake, Pulford & Artymiuk, 1983; Finney, 1979; Boyes-Watson, Davidson & Perutz, 1947). It has also been shown that bound water molecules occupy similar positions with respect to the protein molecule despite the differences in the crystal packing and the composition of salt ions (Blake, Pulford & Artymiuk, 1983). Thus at least

some of these water molecules appear to be an integral part of the protein structure. The bulk water, on the other hand, is highly disordered and fills crystal interstices. The number of molecules of bulk water varies from crystal to crystal for a given protein. It is presumably some of these water molecules that are removed from the crystal in transformations caused by a reduction in the humidity of the environment. However, drastic changes in the volume, and perhaps the organization of bulk water, caused by such transformations are most unlikely to leave the contiguous ordered water molecules undisturbed. The effects of dehydration are therefore likely to be transmitted to the protein molecule through disturbances in ordered water molecules. These disturbances in ordered water structure around protein molecules should be of considerable interest in relation to protein–solvent interactions. Thus, detailed studies on water-mediated transformations in protein crystals, including structure determinations of low-humidity forms, appear to be of considerable value not only in elucidating probable conformational transitions in protein molecules but also in exploring the structural role of bound water molecules.

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