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Structural transformations in protein crystals caused by controlled dehydration

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Abstract. Recent experiments in this laboratory on structural transformations caused by controlled dehydration of protein crystals have been reviewed. X-ray diffraction patterns of the following crystals have been examined under varying conditions of environmental humidity in the relative humidity range of 100–75 %: a new crystal form of bovine pancreatic ribonuclease A grown from acetone solution in tris buffer (I), the well-known monoclinic form of the protein grown from aqueous ethanol (II), the same form grown from a solution of 2-methyl pentan-2,4-diol in phosphate buffer (III), tetragonal (IV), orthorhombic (V), monoclinic (VI) and triclinic (VII) hen egg white lysozyme, porcine 2 Zn insulin (VIII), porcine 4 Zn insulin (IX) and the crystals of concanavalin A(X). I, II, IV, V and VI undergo one or more transformations as evidenced by discontinuous changes in the unit cell dimensions, the diffraction pattern and the solvent content. Such water-mediated transformations do not appear to occur in the remaining crystals in the relative humidity range explored. The relative humidity at which the transformation occurs is reduced when 2-methyl pentan-2,4-diol is present in the mother liquor. The transformations are affected by the crystal structure but not by the amount of solvent in the crystals. The X-ray investigations reviewed here and other related investigations emphasize the probable importance of water-mediated transformations in exploring hydration of proteins and conformational transitions in them.

Keywords. Water-mediated transformations; environmental humidity; protein crystals; conformational transitions; protein hydration.

Introduction

Proteins, as indeed almost all other biomolecules, function in aqueous media. The structural stability of most proteins can be maintained only when they are surrounded by water. Nearly half the volume of even protein crystals is usually occupied by water the removal of which leads to loss of crystallinity. Thus water plays a preeminent role in the structure and action of proteins. Therefore, not surprisingly, hydration of proteins has received considerable attention over the years (Kuntz and Kauzmann, 1974; Finney, 1979; Careri *et al.,* 1980; Blake *et al.,* 1983). High resolution X-ray refinement of the crystal structures of small or medium sized proteins have led to the elucidation in a few cases of water structure around protein molecules; a few water molecules are also found to form an integral part of the protein structure (Blake *et al.,* 1983; Teeter, 1984; Sakabe *et al.,* 1980; Watenpaugh *et al.,* 1979). Thus hydration of proteins is no longer a vague concept; it can be visualized in electron-density maps of sufficiently high resolution. Yet, the precise structural and functional role of protein-water interactions has proved elusive so far. Controlled hydration or dehydration of non-crystalline

Abbreviations used: MPD, 2-Methyl pentan-2,4-diol; r.h., relative humidity.

protein samples has been employed for studying this role using physicochemical and biochemical techniques (Careri *et al.,* 1980; Baker *et al.,* 1983; Poole and Finney, 1983). The first and the only studies of this nature on protein crystals were those carried out on haemoglobin in the late forties and early fifties (Boyes-Watson *et al.*, 1947; Huxley and Kendrew, 1953). These studies were primarily aimed at determining the overall size and shape of the molecule as well as the phase angles of some special X-ray reflections. Protein crystallography then was at its infancy and, not surprisingly, the possible ramifications of the observed results were not further explored.

Our interest in the problem of hydration of proteins and its consequences arose out of the accidental discovery of a structural transformation caused by dehydration in a new crystal form of bovine ribonuclease A (Salunke *et al.,* 1984). Subsequently, different crystal forms of bovine ribonuclease A and hen egg white lysozyme, porcine 2 Zn insulin, porcine 4 Zn insulin and the crystals of concanavalin A were systematically examined under varying conditions of environmental humidity (Salunke, D. M., Veerapandian, B., Kodandapani, R. and Vijayan, M., unpublished results). The results of this examination, reviewed here, appear to be highly relevant to the study of hydration of proteins and conformational transitions in them.

Occurrence and nature of water-mediated transformations

The following crystals were examined under different humidity conditions.

(I) A new monoclinic crystal form of bovine ribonuclease A grown from an acetone solution in tris buffer (Salunke *et al.,* 1984).
(II) The well-known monoclinic form of bovine ribonuclease A grown from aqueous ethanol (Carlisle *et al.,* 1974).
(III) The same form as the above grown from a solution of 2-methyl pentan 2,4-diol (MPD) in phosphate buffer (Kartha *et al.,* 1967).
(IV) Tetragonal hen egg white lysozyme (Blake *et al.,* 1965).
(V) Orthorhombic hen egg white lysozyme (Artymiuk *et al.,* 1982).
(VI) Monoclinic hen egg white lysozyme (Hogle *et al.,* 1981).
(VII) Triclinic hen egg white lysozyme (Moult *et al.,* 1976).
(VIII) Porcine 2 Zn insulin (Adams *et al.,* 1969).
(IX) Porcine 4 Zn insulin (Bentley *et al.,* 1976).
(X) Concanavalin A (Hardman *et al.,* 1971).

The crystals were grown using methods described in the literature (Salunke *et al.,* 1984; King *et al.,* 1956; Steinrauf, 1959; Hogle *et al.,* 1981; Harding *et al.,* 1966; Hardman *et al.,* 1971). X-ray photographs of 15° precession were recorded from the crystals at relative humidities (r.h.'s) of 100 %, 98 %, 95 %, 93 %, 90 %, 88 %, 84 %, 79·5 %, 75% and 66 %. The r.h.'s of the crystal environment were maintained at the desired values by introducing appropriate saturated salt solutions in the glass capillaries containing the crystals (Rockland, 1960). The water content of the crystals at different values of r.h. was estimated using Matthews' method (Matthews, 1968) assuming the partial specific volume to be 0·74 in all cases.

Of the crystals examined, I, II, IV, V and VI undergo one or more reversible transformations, as evidenced by abrupt changes in the unit cell dimensions, the

diffraction pattern, and the solvent content, when the r.h. is reduced in a controlled manner in the range 100–75 %. These water-mediated transformations are clearly distinguishable from the normal drying process in that the quality of the diffraction patterns remains nearly the same above and below the transformations, as can be seen from figures 1 and 2. As indicated earlier, each transformation is accompanied by an abrupt reduction in the amount of solvent in the unit cell. The variations of the solvent content as a function of r.h. in the different crystals that transform are illustrated in figures 3 and 4. It must also be emphasized that the changes in the solvent content are not caused by uniform changes in unit cell dimensions. The changes in cell dimensions are highly anisotropic. The number of protein molecules in the unit cell remains the same throughout the r.h. range of 100–75 % in four out of the five crystals that transform. Monoclinic lysozyme, however, contains four molecules in the high humidity form whereas it contains only two molecules in the low humidity form. The arrangement of the two crystallographically independent molecules in the native crystals is such that the unit cell is pseudo B-centred. After transformation at low humidity, the two molecules become equivalent and consequently the cell truly B-centred. The B-centred cell can be readily transformed into a primitive cell with half the volume.

Five (III, VII, VIII, IX and X) out of the ten crystals examined do not appear to transform in the r.h. range of 100–75 %. Thus, water-mediated transformation in protein crystals, though wide spread, is not perhaps a universal phenomenon. Of the five crystals that do not transform, 2 Zn insulin loses crystallinity at about r.h. 88 %. The unit cell dimensions and the diffraction pattern of each of the remaining four do not change substantially even when the r.h. is reduced to 75 %. The patterns, however, nearly vanish at 66 % r.h.

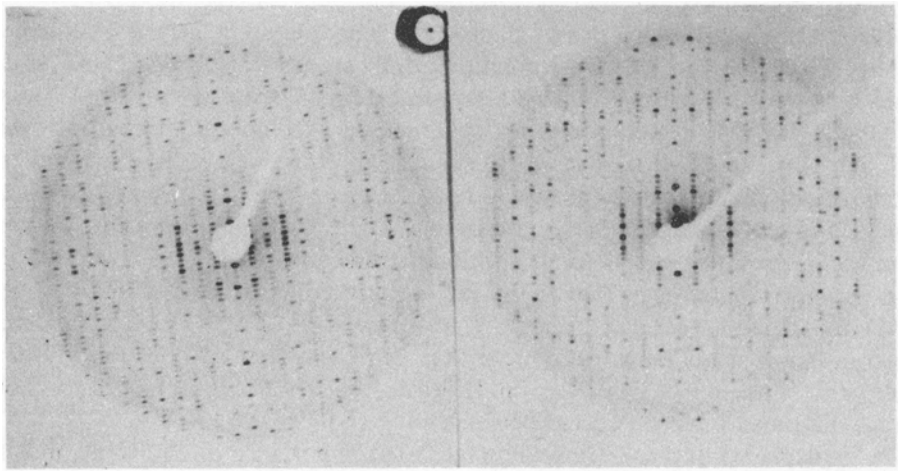

Figure 1. 15° okl precession photographs from the new crystal form of ribonuclease A at (a) 100 % and (b) 93 % r.h.

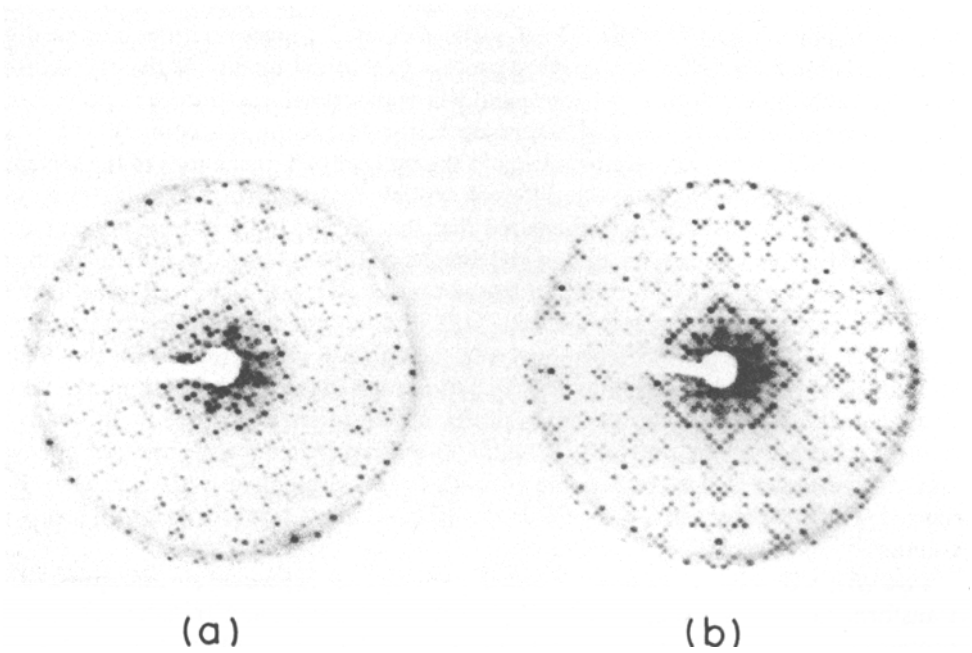

Figure 2. 15° hko precession photographs from tetragonal lysozyme at (a) 100 % and (b) 90 % r.h.

Effect of solvent composition

It is interesting to note that the crystal form of ribonuclease A grown from aqueous ethanol (II) undergoes two transformations, one between 93 % and 90 % r.h. and the other between 88 % and 84 % r.h. But, the same crystal form grown from 2-methyl pentan-2,4-diol (MPD) solution in phosphate buffer (III) does not transform even at 75 % r.h. However, II soaked in MPD solution in phosphate buffer for a few days, behave exactly like III when the humidity of the environment is varied. Likewise, III soaked in aqueous ethanol behaves exactly like II. The crystals of ribonuclease A grown from acetone solution in Tris buffer normally transform between 93 % and 90 % r.h. However, the same crystals soaked in MPD solution in phosphate buffer or aqueous MPD, transform between 88 % and 84 % r.h. The effect of MPD on water-mediated transformations is not confined to the crystals of ribonuclease A. Tetragonal lysozyme transforms between 93 % and 90 % r.h. However, when MPD is present in the mother liquor, the transformation occurs between 84 % and 79.5 % r.h.

The experiments outlined above emphasize the importance of cosolvents in water-mediated transformations. In particular, the presence of MPD in the mother liquor causes the lowering of the r.h.'s at which the transformation occurs. Thus, MPD appears to help protein molecules to retain the water of hydration, in conformity with the earlier observation of the preferential hydration of ribonuclease A in the presence of MPD (Pittz and Bello, 1971; Pittz and Timasheff, 1978). The diffractions patterns,

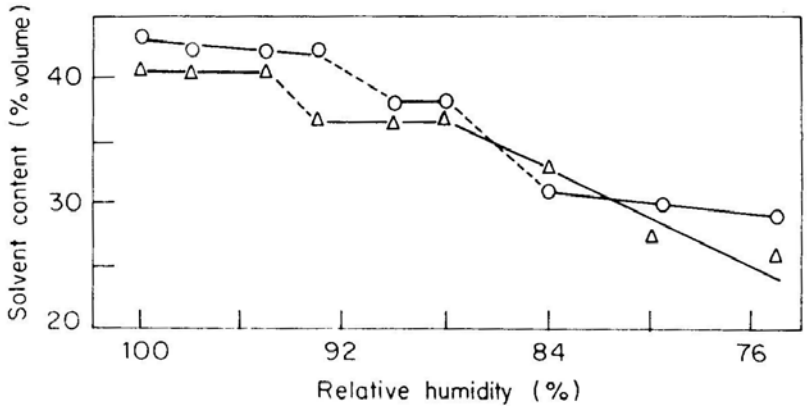

Figure 3. Variation of solvent content as a function of relative humidity in the new crystal form (Δ) and the well-known crystal form grown from aqueous ethanol (O) of ribonuclease A.

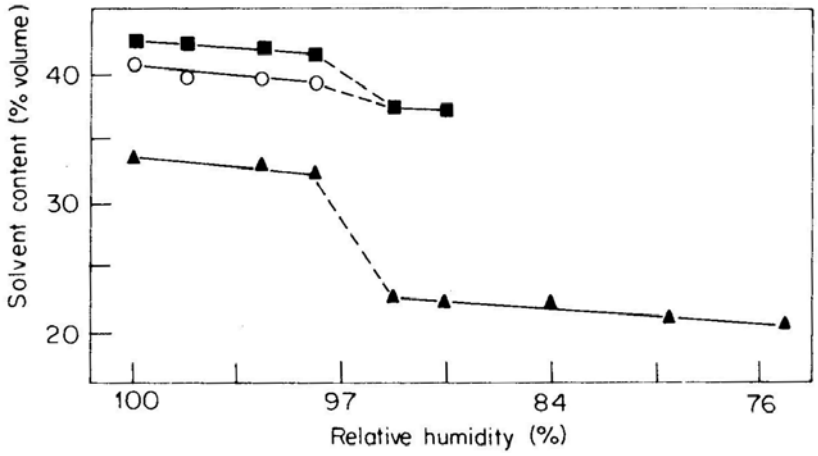

Figure 4. Variation of solvent content as a function of relative humidity in tetragonal (O), orthorhombic (■) and monoclinic (▲) lysozyme.

however, indicate that MPD does not have any significance effect on the crystal structure before or after transformation.

The fraction of the volume of the unit cell occupied by solvent, varies considerably in the crystals examined. This, however, does not appear to be a critical factor affecting water-mediated transformations. For example, tetragonal and orthorhombic lysozyme have a much higher solvent content than monoclinic lysozyme. But all the three forms transform in the same humidity range.

Effect of crystal structure

Water-mediated transformations appear to have a definite dependence on crystal structure. The new crystal form of ribonuclease A (I) soaked in MPD solution in

phosphate buffer transforms between 88 % and 84 % r.h. The monoclinic form grown from MPD solution in phosphate buffer (III) or the same form grown from aqueous ethanol (II) and soaked in MPD solution in phosphate buffer, does not transform even at 75 % r.h. This difference is presumably caused by the difference between the two crystal structures, as the protein and the solvent compositions are the same in the two cases. Similarly, monoclinic and triclinic lysozyme are obtained from the same mother liquor and have comparable solvent content. Yet, monoclinic lysozyme transforms between 93 % and 90 % r.h. whereas triclinic lysozyme does not transform in the r.h. range of 100–75 %

Implications to studies on protein hydration and conformational transitions

It was realised even in the early days of protein crystallography that the water present in protein crystals could be divided into bound water and free or bulk water (Boyes-Watson *et al.*, 1947). Recent high resolution X-ray studies have confirmed that this is indeed the case (Blake *et al.*, 1983; Teeter, 1984). The bound water molecules, as the name implies, are in contact with protein molecules; some of them are internal to the protein and form an integral part of its structure. The number of bound water molecules and the positions they occupy appear to be a characteristic structural feature of the concerned protein (Blake *et al.,* 1983). The amount of bulk water, which fills crystal interstices, however, varies substantially from crystal to crystal. Presumably, the change in water content in water-mediated transformations represents a change in the amount of bulk water. As the bulk water is contiguous to bound water molecules, it is most likely that the changes in the former affect the organisation of the latter. Therefore, the transformations are likely to affect, perhaps indirectly, the water structure around protein molecules. Thus a detailed study of the crystal structures of a given protein before and after transformation should provide useful information on the changes in the hydration shell around the protein molecule caused by the changes in the amount and the composition of bulk solvent.

The observed changes in the unit cell dimensions and the diffractions patterns during water-mediated transformations could have resulted from changes in crystal packing, conformational changes or both. In view of the current interest in the flexibility of proteins (Frauenfelder *et al.*, 1979; Artymiuk *et al.*, 1979; Huber, 1979; Wagner and Wuthrich, 1982; Wagner, 1983; Ribeiro *et al*., 1983; Vijayan and Salunke, 1984), it is the probable conformational changes during these transformations that deserve particular attention. In this context, it is interesting to note that the crystal transformation between 2 Zn insulin and 4 Zn insulin caused by changes in the ionic strength of the mother liquor (Bentley *et al.,* 1978) and the transformation in adenylate kinase resulting from changes in the pH of the environment (Sachsenheimer and Schulz, 1977), produce alterations in the unit cell dimensions and the diffraction pattern comparable to those observed in water-mediated transformations. These transformations have been shown to involve substantial conformational changes. The same is thus likely to be true about water-mediated transformations also. Recent hydrogen exchange, nuclear magnetic resonance and other spectroscopic studies also indicate that changes in hydration are accompanied by conformational changes (Poole and Finney, 1984; Baker *et al.*, 1983;

Careri *et al.*, 1980). The occurrence of conformational changes could be discerned from preliminary results themselves in one of the crystals examined here. The high humidity form of monoclinic lysozyme contains two crystallographically independent molecules which are known to exhibit conformational differences (Rao *et al.*, 1983). As mentioned earlier, the molecules become equivalent in the low humidity form. This should obviously involve conformational changes in at least one, and possibly both, of the molecules. The balance of evidence thus appears to favour the probability of conformational transitions occurring in water-mediated transformations. An elucidation of the nature of such transitions, if they occur, should await high resolution X-ray analysis of protein crystals before and after the transformation.

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