WATER-MEDIATED STRUCTURAL TRANSFORMATIONS IN A NEW CRYSTAL FORM OF RIBONUCLEASE A AND TETRAGONAL LYSOZYME

DINAKAR M. SALUNKE, B. VEERPANDIAN and M. VIJAYAN
Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

ABSTRACT

A new form of ribonuclease A crystals, space group $P2_1$ with $a = 33.9$, $b = 106.3$, $c = 31.8$ Å, $\beta = 98.5°$ and $z = 4$, was prepared by slow diffusion of acetone into a protein solution in tris buffer at, pH 7.6. The crystals undergo a structural transformation, as evidenced by changes in the x-ray diffraction pattern and the unit cell dimensions, when the relative humidity of the environment is varied systematically. Transformations of this type are found to occur in the well known tetragonal crystal form of lysozyme also. Such water-mediated transformations, which could well occur in a variety of protein crystals, provide a useful handle for exploring possible conformational transitions and the general problem of hydration of proteins.

INTRODUCTION

THE crystal structures of well over hundred different globular proteins have been determined during the last two decades and they have provided a wealth of information on protein architecture and structure-activity relationships. In recent years, however, efforts are underway not only to determine the structures of new proteins but also to investigate the flexibility of proteins with known structures. Many recent crystallographic[1-4] and spectroscopic[5-7] studies indicate that globular proteins, though endowed with unique tertiary structures, possess some conformational flexibility which is often related to biological function.

Another related aspect of protein structure which has received considerable attention pertains to the hydration of proteins. Proteins almost invariably exist and act in aqueous medium. Nearly half the volume of even protein crystals is usually made up of water and they can be considered as concentrated solutions in relation to solvent-protein interactions. Recent high resolution x-ray analyses of proteins have indeed provided valuable information on protein-water interactions and ordered water structure in the solvent regions in the crystals[8,9]. A wide variety of other techniques have also been used in attempts to understand hydration of proteins with particular reference to the influence of water on protein structure and function[10].

In the context of the growing interest in the conformational flexibility and the hydration of proteins, outlined above, our recent observation of water-mediated transformations in the crystals of two well known proteins, reported here, appears to be of considerable importance. During attempts to co-crystallize ribonuclease A with other proteins in order to study protein-protein interactions as well as the conformational variability of the concerned protein molecules, we obtained a new crystal form of ribonuclease A. It was observed that loss of mother liquor in the glass capillaries containing these crystals, beyond a critical value resulted in significant changes in the diffraction pattern accompanied by a reduction in the unit cell volume, although the quality of the pattern remained unchanged. This observation led us to a systematic investigation of the changes, as a function of the relative humidity of the environment, in the diffraction patterns from the new crystal form of ribonuclease A and the well known tetragonal crystal form of hen egg white lysozyme. The results of this investigation are indicative of the high potential of this approach in exploring possible conformational transitions in proteins as a result of changes in their aqueous environment.

EXPERIMENTAL

Bovine pancreatic ribonuclease A and Hen egg white (HEW) lysozyme were obtained commercially from Sigma Chemical Company. The new form of ribonuclease A was crystallized by slow diffusion of acetone into a protein solution in tris buffer at pH 7.6. The crystals were characterised using precession photographs. Tetragonal lysozyme crystals were prepared by the method described in the literature[11]. Diffraction patterns were obtained at different relative humidity conditions by introducing appropriate saturated salt solutions in the glass capillaries containing the crystals[12].

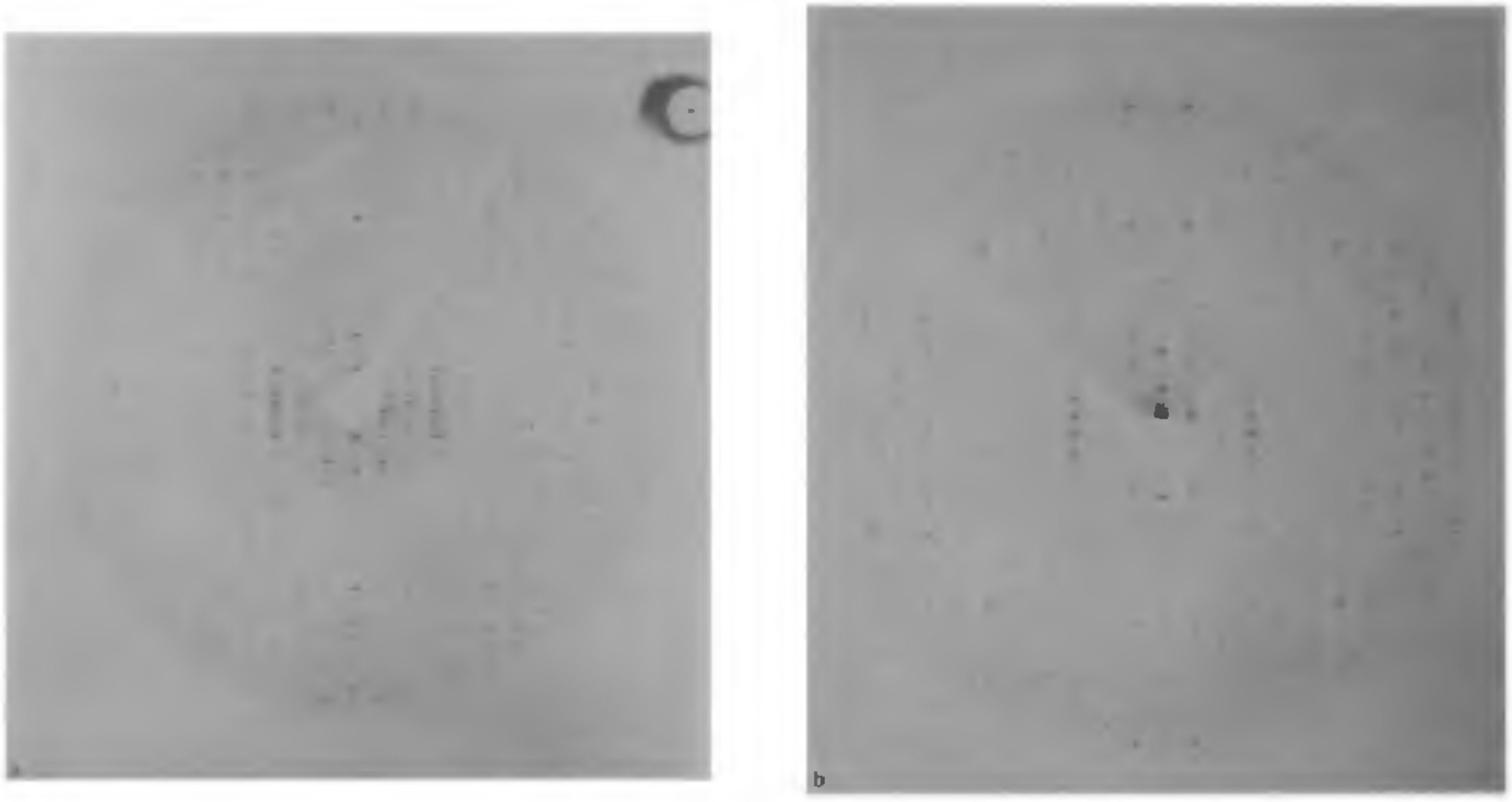

Figure 1. okl 15° precession photographs from the new crystal form of ribonuclease A at **(a)** 100% and **(b)** 90% relative humidity.

RESULTS

The new crystal form of ribonuclease A belongs to the monoclinic space group $P2_1$ with $a = 33.9$, $b = 106.3$, $c = 31.8$ Å and $\beta = 98.5°$. Comparison with other known forms of the protein indicates two protein molecules to be present in the asymmetric unit of the new form with a solvent content of 40.7% by volume.

The diffraction patterns from the crystals obtained in the relative humidity range of 100 to 95% are almost identical to that obtained in the environment of the mother liquor. As can be seen from figure 1, the pattern changes significantly when the relative humidity is reduced to 93%. The pattern remains the same in the range of 93 to 88% relative humidity. Further reduction of humidity results in the slow deterioration of the diffraction pattern which almost disappears below a relative humidity of about 44%. Thus a structural transformation takes place between 95 and 93% relative humidity. The changes in the unit cell volume parallel those in the diffraction pattern, as can be seen from figure 2. The cell volume remains almost constant in the 100 to 95% relative humidity range. It decreases sharply between 95 and 93% relative humidity with another constant region between 93 and 88% relative humidity. The cell volume decreases continuously with further decrease in humidity. The transformation that takes place between 95 and 93% relative humidity is completely reversible in terms of the nature of the diffraction pattern and the cell volume. The quality of the pattern remains good in

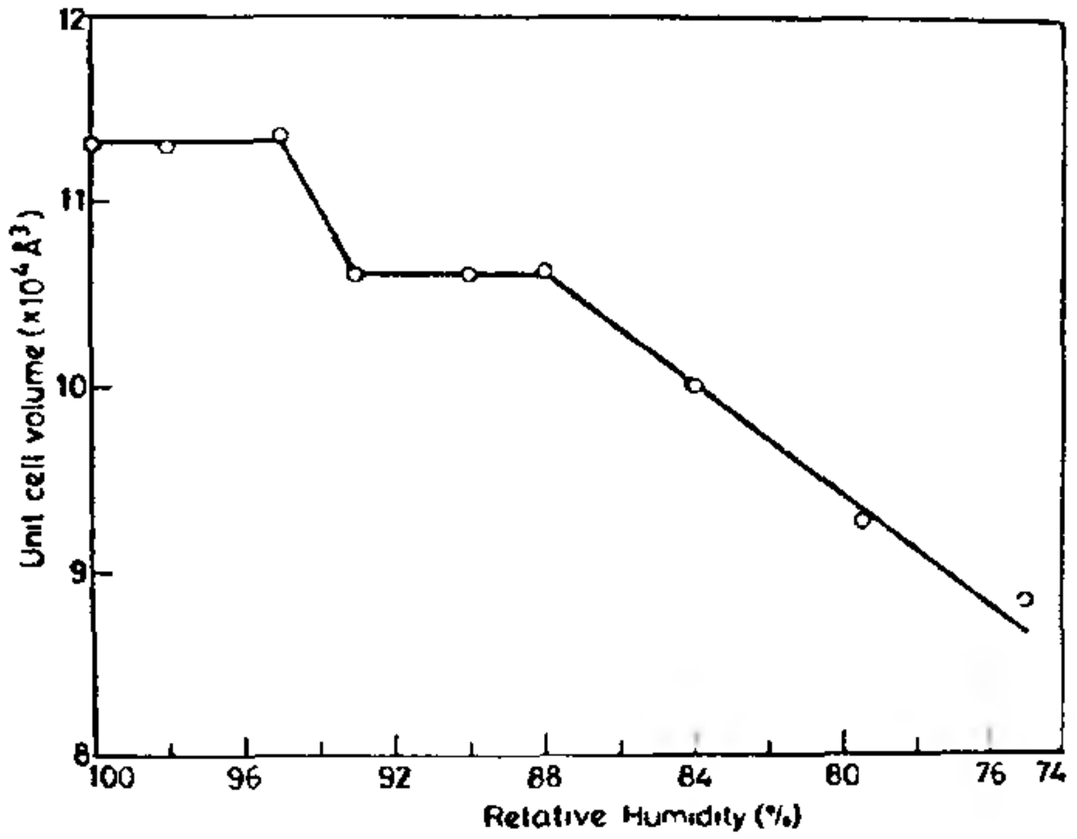

Figure 2. Variation of unit cell volume of the new form of ribonuclease A as a function of humidity.

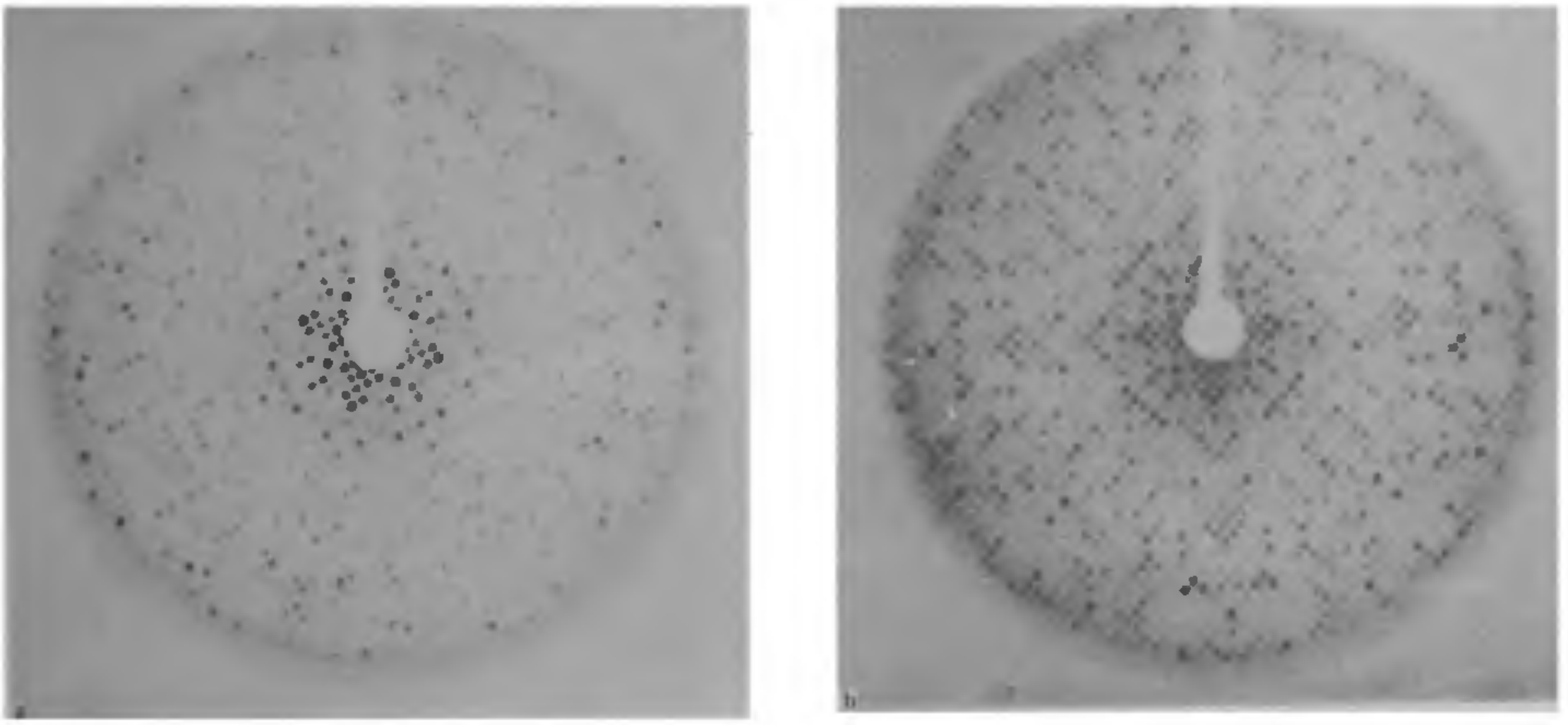

Figure 3. hko 15° precession photographs from tetragonal HEW lysozyme at (a) 100% and (b) 90% relative humidity.

the relative humidity range of 100 to 88%. However, once a crystal is kept at a relative humidity much below 88%, the quality of the pattern is not completely regained even when the relative humidity of the environment is raised to a value above 88%.

Other well known crystal forms of ribonuclease A are also being examined for water-mediated structural transformations. Preliminary results of this examination indicate that no transformation takes place in the crystals grown in the presence of 2-methylpentan-2,4-diol (MPD)[13] even when the relative humidity is reduced to a value as low as 75%.

Water-mediated transformations occur in tetragonal HEW lysozyme also as can be seen from figure 3 which compares the diffraction patterns of the crystals at 100% and 90% relative humidity. Figure 4 illustrates the variation of cell volume as a function of relative humidity. The diffraction pattern from these crystals, however, deteriorates more rapidly than that from the crystals of ribonuclease A; a good pattern was observed at a relative humidity of 88%, but not at 84% or below. The patterns obtained at relative humidities of 100% and 98% were the same as that observed from native crystals in the presence of the mother liquor. The crystals gave similar patterns at 95% and 93% relative humidity, but they were

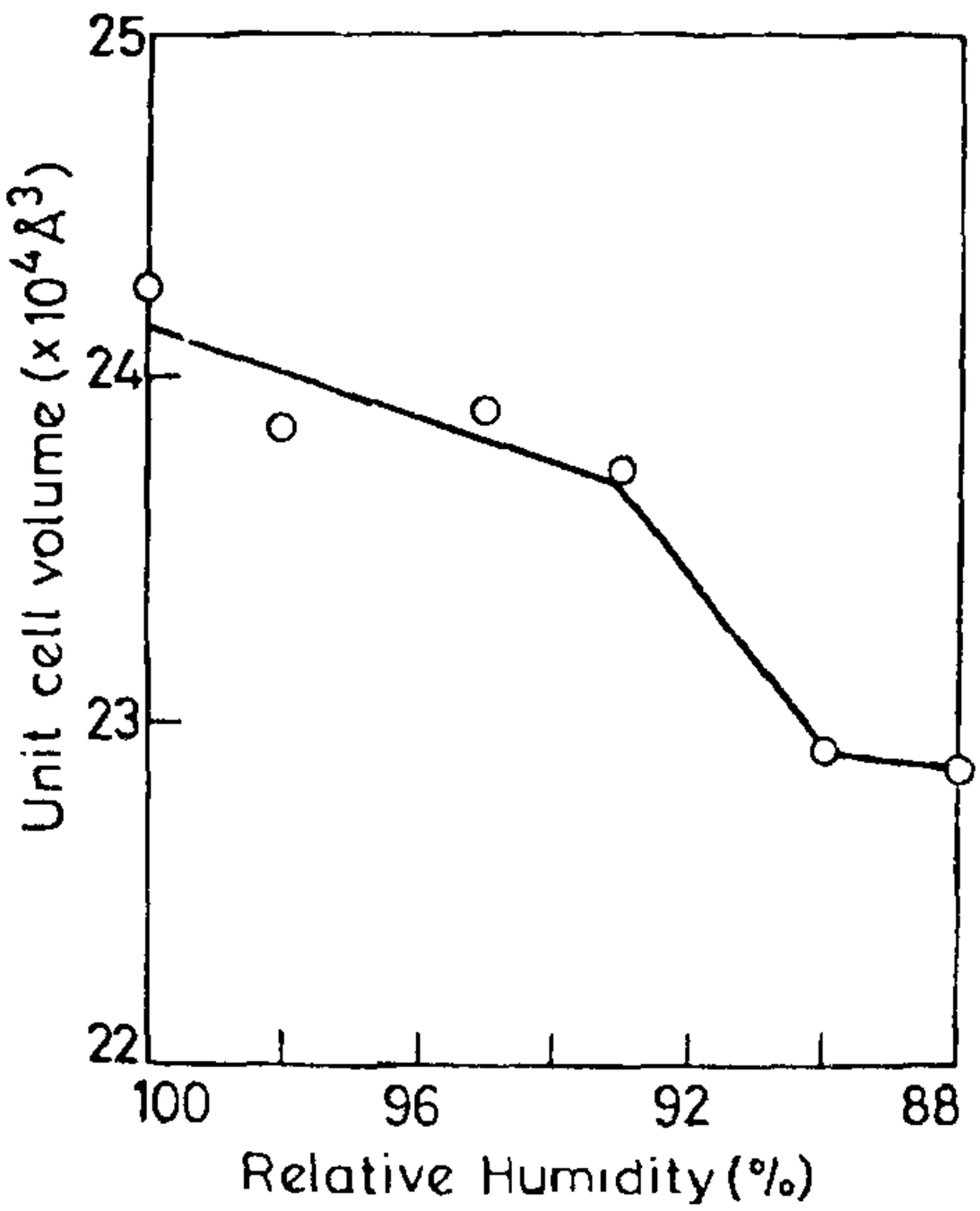

Figure 4. Variation of unit cell volume of tetragonal HEW lysozyme as a function of humidity.

somewhat different from those obtained at 100 % and 98 %. Still more, marked changes were observed in the patterns obtained at relative humidities of 90 % and 88 %, although the two patterns were similar between themselves. Thus, at least two transformations appear to occur between 100 % and 88 % relative humidity. Although the diffraction pattern vanishes at low relative humidities, it almost regains its original quality when the relative humidity is raised to 100 % or 95 %.

DISCUSSION

The first and, as far as we are aware, the only other systematic investigation on the effect of humidity on protein crystals was carried out on haemoglobin way back in the late forties and the early fifties[14–16]. The results were then interpreted as due to the movement of layers of protein molecules and used for the phase determination of some low angle reflections. Protein crystallography was then at its infancy and, not surprisingly, further probable implications of water-mediated transformations were not seriously explored. Our results point to the probability of water-mediated transformations being a phenomenon likely to occur in a variety of protein crystals. This transformation could consist of changes in crystal packing, conformational changes or, most probably, both. Obviously, it is the probable conformational changes that deserve serious attention.

As indicated earlier, a substantial amount of work, using techniques other than crystallography, has already been carried out on the possible effects of change in water content on protein conformation. The results of the earlier work of this nature were interpreted as indicative of significant effects of dehydration on protein conformation[10]. A subsequent detailed examination of heat capacity, spectroscopic properties, diamagnetic susceptibility and enzyme activity of lysozyme powders as a function of hydration level led to a hydration model which does not involve significant changes in protein conformation[17]. More recent studies based on hydrogen exchange and nuclear magnetic resonance and laser Raman spectroscopy, clearly indicate that change in hydration is accompanied by conformational changes[18, 19]. Also, a recent analysis of the environments of α-helices in different protein structures has led to the conclusion that water interactions could lead to significant perturbations in secondary structures[20].

To summarise, the results from a variety of physico-chemical and biochemical work on hydration of proteins and our preliminary x-ray studies appear to indicate the probability of the occurrence of cooperative water-mediated conformational transitions in protein molecules. It is felt that water-mediated transformations in protein crystals, like those reported in this communication, provide a useful handle for exploring these transitions as well as the general problem of hydration of proteins. Our current efforts in this regard include a detailed examination of water-mediated transformation in different crystal forms of ribonuclease A, lysozyme and other proteins with known structures. Efforts directed towards the high resolution x-ray analysis of the low humidity form of tetragonal lysozyme and the new crystal form of ribonuclease A in its two hydration states, are also in progress.

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1. Frauenfelder, H., Petsko, G. A. and Tsernoglou, D., *Nature* (*London*) 1979, **280,** 558.
2. Artymiuk, P. J., Blake, C. C. F., Grace, D. E. P., Oatley, S. J., Phillips, D. C and Sternberg, M. J. E., *Nature* (*London*) 1979, **280,** 563.
3. Blow, D. M., In *Ciba Foundation Symposium 60* (New series), 1978, p. 55.
4. Huber, R., *Trends Biochem. Sciences*, 1979, **4,** 271.
5. Wagner, G. and Wuthrich, K., *J. Mol. Biol.*, 1982, **160,** 343.
6. Wagner, G., *Q. Rev. Biophys.*, 1983, **16,** 1.
7. Ribeiro, A. A., King, R. and Jardetzky, O., In *Conformation in Biology* (eds.) R. Srinivasan, and R. H. Sarma, Adenine Press, New York, 1983, p. 39.
8. Blake, C. C. F., Pulford, W. C. A. and Artymiuk, P. J., *J. Mol. Biol.* 1983, **167,** 693.
9. Finney, J. L., *Phil. Trans. R. Soc.* (*London*), 1977, **278,** 3.
10. Kuntz Jr., I. D. and Kauzmann, W., *Adv. Protein Chem.*, 1974, **28,** 239.
11. Alderton, G. and Fevold, H. L., *J. Biol. Chem.*, 1946, **164,** 1.

12. Rockland, L. B., *Anal. Chem.* 1960, **32,** 1375.
13. Kartha, G., Bellow, J. and Harker, D., *Nature* (*London*), 1967, **213,** 862.
14. Boyes-Watson, J., Davidson, E. and Perutz, M. F., *Proc. R. Soc.* (*London*), 1947, **A191,** 83.
15. Huxley, H. E. and Kendrew, J. C., *Acta Crystologra.*, 1953, **6,** 76.
16. Bragg, L. and Perutz, M. F., *Proc. R. Soc.* (*London*), 1952, **A213,** 425.
17. Careri, G., Gratton, E., Yong, P. H. and Rupley, J. A., *Nature* (*London*) 1980, **284,** 572.
18. Baker, L. J., Hansen, A. M. F., Bhaskara Rao, P. and Bryan, W. P., *Biopolymers*, 1983, **22,** 1637.
19. Poole, P. L. and Finney, J. L., *Biopolymers*, 1983, **22,** 255.
20. Blundell, T., Barlow, D., Borkakoti, N. and Thornton, J., *Nature* (*London*) 1983, **306,** 281.

SYNTHESIS OF NEW 5,3/5 AND 2-SUBSTITUTED (1,3,4)-OXADIAZOLES AND THEIR RELATED PRODUCTS AS POTENTIAL ANTIFUNGAL AND ANTIVIRAL AGENTS

NEERU SRIVASTAVA, SURENDRA BAHADUR, H. N. VERMA* and M. M. ABID ALI KHAN
*Department of Chemistry, *Plant Virus Laboratory, Lucknow University, Lucknow 226 007, India.*

ABSTRACT

Three 5-(6′,8′-disubstituted-2′-ethyl-quinazolin-4'-oxymethyl)-2-mercapto-1,3,4-oxadiazoles (III) were synthesised by the cyclisation of N-(6′,8′-disubstituted-2′-ethyl-quinazolin-4′-oxy-acetyl) hydrazines (I) and two 5-(6′,8′-disubstituted-2′-ethyl-quinazolin-4′-oxy-methyl)-2-pheryl-1,3,4-oxadiazoles (II) were prepared by the compound (I) in the presence of $POCl_3$ and benzoic acid. III undergoes condensation with chloro compounds at position-2 to give IV in the presence of dryacetone and anhyd. potassium carbonate. III also undergoes Mannich condensation at position-3 in the presence of formaldehyde and 3/4-(2′-benzimidazolyl)-anilines and furnished V. All the synthesised compounds were screened against plant virus SRV both *in vivo* and *in vitro* for their antiviral action at a conc. 1 mg/ml and found to inhibit 4–48% *in vivo* and 6–53% *in vitro*. Four compounds (III 1, IV 3, V 1 and V 2) were screened for their antifungal action against *Fusarium* sp. and *A. niger* and three compounds caused a very significant inhibition at a conc. 1:1000.

INTRODUCTION

OUR earlier work on the synthesis of N-(6′, 8′-disubstituted -2′ -ethyl-quinazolin-4′- oxy-acetyl)-substituted hydrazines[1] as intermediate for the title compounds revealed that they possessed antimicrobial activity and since oxadiazole has been reported to exhibit significant biological activities[2,3], it was considered worthwhile to incorporate this moiety in the parent nucleus (I) and to evaluate them as fungicides and virucides. Further substituents such as phenyl mercapto, 2-methyl-1-benzimidazolyl, *N*-phenyl-*N*′-acetyl-ureido at position-2 in II and III were introduced to evaluate them for their antifungal and antiviral action. The tautomer of III, which has a labile hydrogen at position-3, was made to undergo Mannich condensation with different benzimidazolyl anilines as benzimidazole and Mannich bases are also well known for their antimicrobial activities[4–7].

2,5-Disubstituted 1,3,4-oxadiazoles (II) have been synthesised by the cyclisation of compound (I) in the presence of phosphorous oxychloride and benzoic acid and I, when cyclised in the presence of carbon disulphide and potassium hydroxide, furnished 5-substituted 2-mercapto-1,3,4-oxadiazoles (III). III undergoes condensation at position-2 with chloro compounds like *N*-phenyl-*N*′-chloroacetyl ureas and 2-chloro methyl-benzimidazole in the presence of dry acetone and anhyd. potassium carbonate and resulted in the formation of IV. III also undergoes Mannich condensation at position-3 in the presence of formaldehyde and benzimidazolyl anilines to give V. Various steps involved in the synthesis of the titled compounds are shown in scheme 1. Structural charac-