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An X-ray Analysis of Native Monoclinic Lysozyme. A Case Study on the Reliability of Refined Protein Structures and a Comparison with the Low-Humidity Form in Relation to Mobility and Enzyme Action

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Abstract

The atomic models of native monoclinic lysozyme obtained by refinement at Bangalore and elsewhere [Young, Dewan, Nave & Tilton (1993). *J. Appl. Cryst.* **26**, 309–319] differed significantly in the flexible regions of the protein molecule. The two models were reconciled starting from regions where they were in reasonable agreement to produce an improved model which yielded an R value of 0.169 for 12816 observed reflections in the 10–2 Å resolution range. The reconciled model was compared with the structure of the 88% relative humidity form obtained through a water-mediated transformation [Madhusudan, Kodandapani & Vijayan (1993). *Acta Cryst.* D**49**, 234–245]. Parts of the flexible regions of the molecule register significant movements during the transformation. The changes resulting from the transformation from the native to the low-humidity forms are pronounced in many of the side chains in the active-site region, thus indicating the relationship between hydration, mobility and enzyme action. The fact that the overall changes in molecular geometry resulting from water-mediated transformation are similar to those which occur during enzyme action, further emphasizes this relationship.

1. Introduction

We have been exploring the variability in protein hydration and its structural consequences, including its implication to enzyme action, using an approach involving water-mediated transformations induced by changes in the solvent content in protein crystals resulting from systematic changes in environmental humidity (Salunke, Veerapandian & Vijayan, 1984; Salunke, Veerapandian, Kodandapani & Vijayan, 1985; Kodandapani, Suresh & Vijayan, 1990; Madhusudan & Vijayan, 1991; Madhusudan, Kodandapani & Vijayan, 1993; Radhakishan, Chandra, Sudarsanakumar, Suguna & Vijayan, 1995; Nagendra, Sudarsanakumar & Vijayan, 1995). This exploration has also led to the elucidation of the nature of mobility in hen egg-white lysozyme and bovine ribonuclease A and the identification of the relatively invariant features in their hydration shell (Madhusudan & Vijayan, 1991; Radhakishan *et al.*, 1995). Among the water-mediated transformations, the one exhibited by monoclinic lysozyme was the most impressive. During the transformation which occurs around a relative humidity of 90% and is accompanied by substantial loss of water, the two crystallographically independent protein molecules in the native form become equivalent. The solvent content of the low-humidity 88% form is as low as 22% which is perhaps the lowest observed in any protein crystal until now. This form diffracts substantially better than the native form and its X-ray analysis has also led to useful information on the water structure associated with proteins (Madhusudan *et al.*, 1993). A detailed comparison of this structure with that of the native form could not be carried out as the available structure of native monoclinic lysozyme was only incompletely refined (Rao, Hogle & Sundaralingam, 1983). Therefore, we undertook a thorough refinement of the structure of the native crystals using data collected on an area detector with the partially refined structure available in the Protein Data Bank (Bernstein *et al.*, 1977) as the starting model. By the time this refinement was completed, another independent refinement of the structure using diffractometer data with the same starting model became available (Young, Dewan, Nave & Tilton, 1993). A comparison of these two refined structures and the procedures adopted for the reconciliation of the differences between them form the subject matter of the first part of this paper.

Problems associated with the refinement of protein structures especially the possibility of arriving at a crystallographically acceptable, but erroneous, refined structure, have been a matter of concern from the early days of protein structure refinement (Chambers & Stroud, 1977; Vijayan, 1980). Several investigations have been carried out to explore these problems through comparisons of the parameters of the same structure obtained through refinement by different groups, different data sets or both. The results of three of the most recent of these investigations (Daopin, Davies, Schlunegger & Grutter, 1994; Harata, 1994; Fields *et al.*, 1994) underline the reliability of refined structures. The results of a fourth

Table 1. *Crystal data and summary of data collection*

Space group	$P2_1$
a (Å)	27.99
b (Å)	62.84
c (Å)	60.36
β (°)	90.9
Z	4
No. of unique reflections measured up to 2 Å resolution	12993
No. of reflections with $I > 2\sigma(I)$ in the 10–2 Å resolution shell	12851
Merging R for all reflections	0.053

Table 2. *Summary of refinement parameters pertaining to initial models 1I and 2I and final models 1F and 2F*

Parameter	1I	2I	1F	2F
R.m.s. deviation in bond distance (Å)	0.016	0.017	0.012	0.018
R.m.s. deviation in angle distance (Å)	0.043	0.033	0.041	0.055
R factor	0.196	0.179	0.169	0.145
No. of reflections	12778	10739	12816	10725
Resolution shell (Å)	10–2	6–1.9	10–2	6–1.9
No. of protein atoms	1986	2002	1995	1992
No. of water O atoms	296	191	266	276

(Ohlendorf, 1994) and the present investigation are somewhat less reassuring and they emphasize the need for further exploration of methods to ensure the reliability of refined protein structures. The present work also enunciates an approach for reconciling the differences between different sets of refined parameters of the same structure. It has been shown that the reconciled model of native monoclinic lysozyme represents an improvement on the two original refined models and it has been used for comparison with the structure of the low-humidity form to investigate the relationship between hydration, mobility and enzyme action.

2. Methods

Hen egg-white lysozyme was obtained commercially from the Sigma Chemical Company. The monoclinic crystals were prepared using the well known method (Steinrauf, 1959) from a solution of 1% protein and 2% $NaNO_3$ in sodium acetate buffer, pH 4.6. Data were collected to a resolution of 2.0 Å on a Siemens area-detector system mounted on a Marconi Avionics rotating-anode X-ray generator and were processed using the *XENGEN* suite of programs (Howard *et al.*, 1987). Statistical information pertaining to the data is given in Table 1. The structure was refined by the Hendrickson–Konnert restrained least-squares program *PROLSQ* (Hendrickson & Konnert, 1980) incorporated in the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994) with the coordinate set 1LYM in the Protein Data Bank (Bernstein *et al.*, 1977) based on partial refinement by Rao *et al.* (1983). All five main-chain atoms were restrained for peptide-group geometry. Protein solvent contacts were not restrained. Full occupancies were used for all atoms. $2F_o - F_c$, $F_o - F_c$ and 'OMIT'-type maps (Vijayan, 1980; Bhat & Cohen, 1984) were used at different stages of refinement. Several rounds of model building with *FRODO* (Jones, 1978) using the maps were carried out in the course of the refinement. Special attention was paid to atoms corresponding to comparatively ill defined regions of the map and those which exhibited substantial deviation when the two molecules in the asymmetric unit were superposed using the method of Rossmann & Argos (1975).

2.1. *Comparison of the two models*

While the structural results obtained from the above refinement were being analysed, the results of the independent refinement of the same structure ($P2_1$; $a = 27.99$, $b = 62.92$, $c = 60.48$ Å, $\beta = 90.67°$) by another group became available (Young *et al.*, 1993). This refinement was based on diffractometer data from crystals grown in the same manner as we did. The final cycles of the refinement of the structure using *PROLSQ* were similar to those carried out by us. The overall results of the two sets of refinement are summarized in the first two columns of Table 2. A careful examination of the two refined models showed that the differences in detail between them were substantially more than what could be reasonably anticipated on the basis of Luzzati analysis (Luzzati, 1952).

In space group $P2_1$, the origin along y can be fixed arbitrarily. Therefore, the y coordinates of the model of Young *et al.*, hereafter referred to as model 2I, were shifted by a constant value in order to make it directly comparable to our model (model 1I). The crystals contain two molecules in the asymmetric unit (A1I and B1I in our model and A2I and B2I in the other model). The r.m.s. deviations among all atoms between A1I and A2I, and B1I and B2I were 0.92 and 0.91 Å, respectively. As is to be expected, the corresponding values for all main-chain atoms were lower at 0.41 and 0.34 Å respectively, while those for all side-chain atoms were higher at 1.25 and 1.26 Å, respectively.

On the basis of the analysis of five well refined structures of lysozyme using difference distance matrices (Nishikawa, Ooi, Isogasi & Saito, 1972; Kundrot & Richards, 1987), we had earlier demonstrated that the lysozyme molecule can be broadly divided into a relatively rigid region made up of 43 residues, moderately flexible regions involving 64 residues and highly flexible regions consisting of 22 residues, as far as the main-chain conformation is concerned (Madhusudan & Vijayan, 1991). On a rough and ready estimate, of the 1034 main-chain atoms in the

two crystallographically non-equivalent molecules, 344 belong to the rigid region, 512 to the moderately flexible regions and 178 to the highly flexible regions. The numbers of main-chain atoms among them that differ in their positions by more than 0.5 Å in the two models are 5, 21 and 29, respectively, accounting for 1.5, 4.1 and 16.3%, of the rigid, moderately flexible and highly flexible regions, respectively. Thus, the differences between the two molecules appear to be primarily in the regions of the molecule which are inherently flexible. The observed differences in the positions of the side-chain atoms also appear to be related to the flexibility of the groups to which they belong. For example, of a total of 226 side-chain atoms the positions of which differ by more than 1 Å in the two models, 108 belong to lysine and arginine, whose long side chains are known to be generally flexible. Furthermore, 82 of these atoms are at the delta position or beyond towards the termini of the side chains. The relationship between differences in atomic positions and displacement parameters, also confirm the higher probability of differences in locating atoms in flexible regions. For instance, the average B value in 1I for protein atoms which deviate by less than 1 Å is 8.3 Å^2 whereas the value for those which deviate by more than 1 Å is 17.8 Å^2.

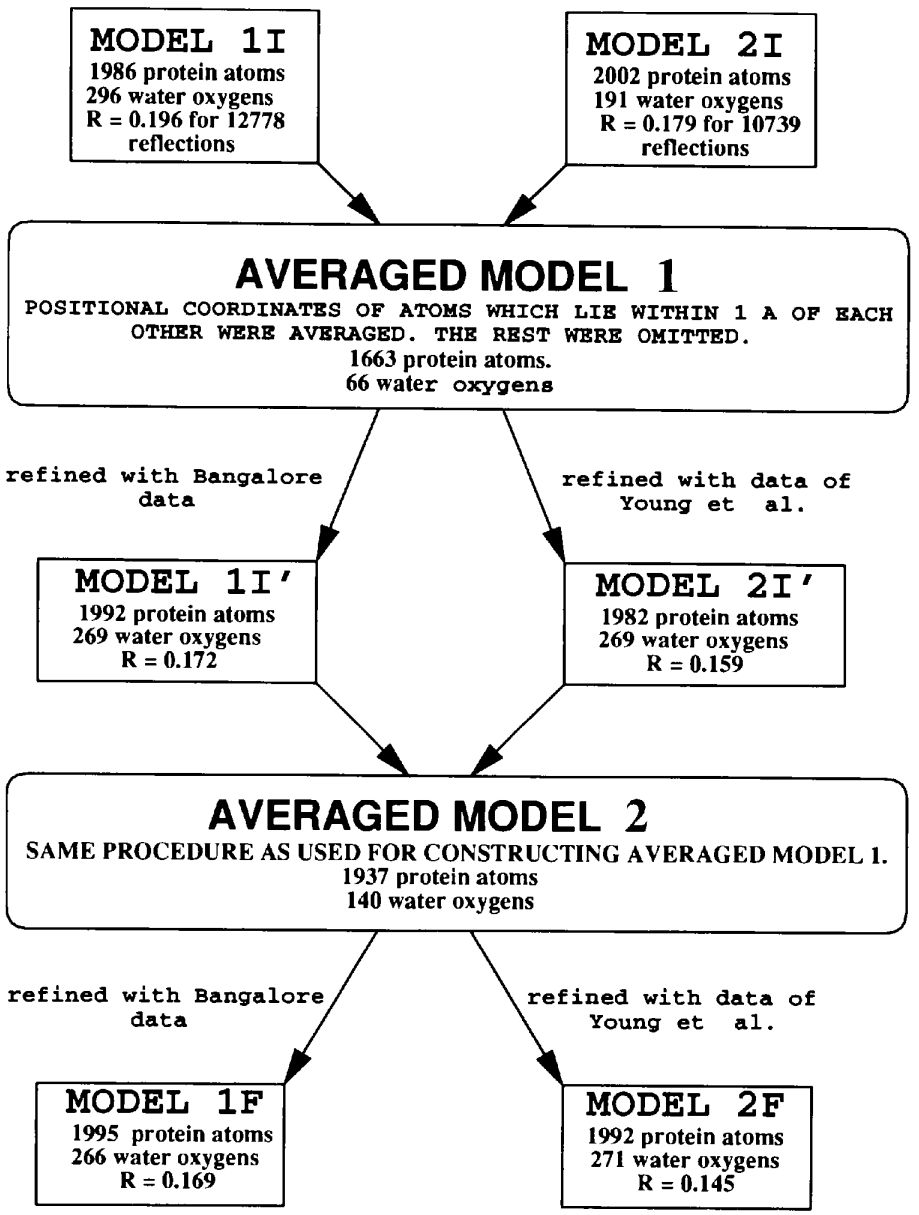

Fig. 1. Outline of the procedure employed for reconciling the two models.

2.2. *Reconciliation of the two models*

The procedure employed for reconciling the two models is outlined in Fig. 1. Atoms which differed in the positions by 1 Å or more were omitted from both the models. A few other atoms were also omitted to avoid unconnected protein atoms in the structure or because their positions appeared uncertain in the electron-density map. The average values of the coordinates were used for the remaining atoms. The model that resulted was regularized using the *NOSF* option in the *CCP*4 package. This averaged model (averaged model 1) was subjected to independent sets of restrained refinement and model-building cycles, one employing the Bangalore data and the other using the data of Young *et al.* (1993). The cycles converged resulting in model 1I′ and model 2I′. The two models were again reconciled to produce the averaged model 2 using the same procedure as that employed to obtain the averaged model 1 from model 1I and 2I. The model was again subjected to two sets of refinement and model-building cycles to yield models 1F and 2F. The results of the final cycles of refinement are summarized in Table 2.

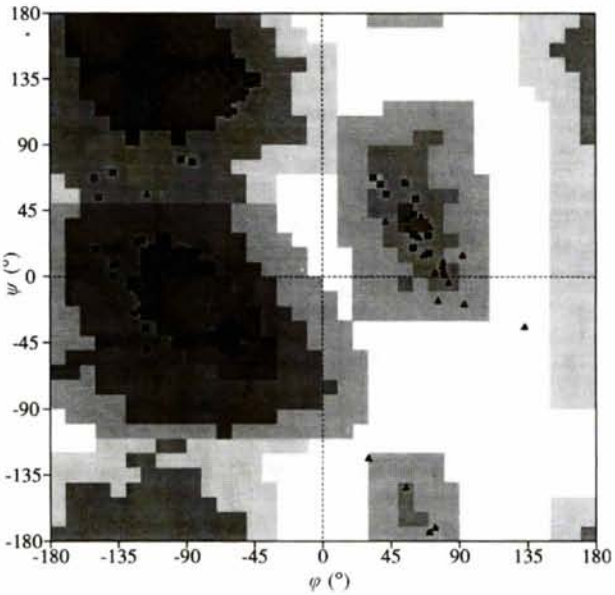

Fig. 2. Ramachandran plot (Ramachandran & Sasisekharan, 1968) of model 1F produced with the program *PROCHECK* (Laskowski, MacArthur, Moss & Thornton, 1993).

The reconciliation procedure could have been carried further, but it was not felt necessary as models 1F and 2F appeared to be as close to each other as could be reasonably expected. The r.m.s. difference between the two models for protein atoms was 0.31 Å. The corresponding differences for the main-chain and the side-chain atoms were 0.15 and 0.48 Å, respectively. The number of water molecules which differed by less than 1 Å in the two models was 169, a number much higher than the number of such water molecules in averaged model 2. The quality of the two final models appeared to be similar. Model 1F yielded a higher R factor than model 2F. However, the number of reflections used in the refinement of the former was greater than that in the latter. There are altogether 10400 reflections common to the two data sets. The refinement of the model 1F against these reflections yielded an R factor of 0.149 while that of model 2F against the same reflections in the data set of Young *et al.* gave an R factor of 0.136. The two R values are not very different. In fact the two data sets are very similar despite the fact that they were collected by different methods from different crystals. The $R_{\rm merge}$ between them was 0.072, a value close to, though higher than, the $R_{\rm merge}$ of the Bangalore data (Table 1).

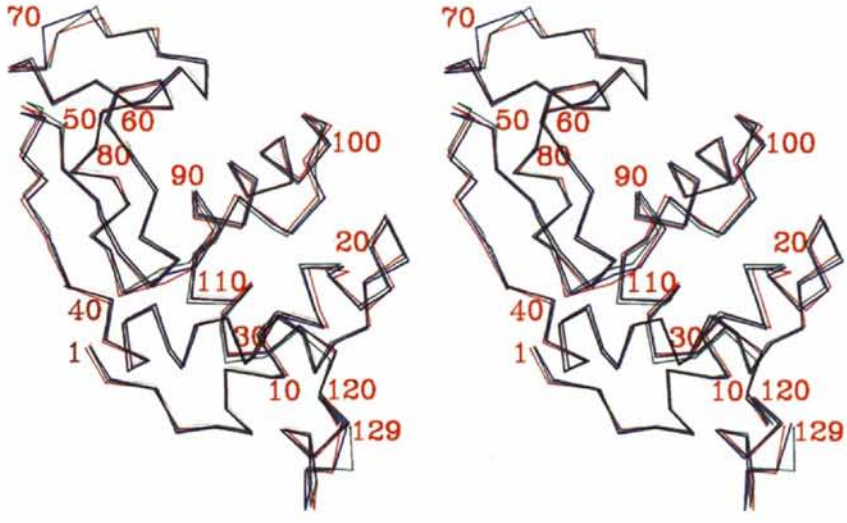

Fig. 3. Stereoview of the superposition of the C^{α} atoms of molecules A (blue) and B (green) and the molecule in the low-humidity form (red).

3. Results and discussion

3.1. *Reliability of the final models*

It is important to investigate if the final reconciled models, model 1F and model 2F, represent an improvement on the original models, model 1I and model 2I, in terms of accuracy. The R factor corresponding to model 1F is lower than that corresponding to model 1I for the same data set; the same is true about model 2F and model 2I. This indicates that the final models are more precise, but not necessarily more accurate.

The water molecules, whose positions were not restrained during refinement and are determined independently of any starting model, perhaps provide a means of assessing the relative reliability of the refined structures. We had earlier shown that 30 water molecules remain invariant in the hydration shells of the molecules in the five well refined structures of hen egg-white lysozyme (Madhusudan & Vijayan, 1991). A water molecule was considered invariant with respect to a pair of structures if it interacts with at least one common protein atom in the two structures and if, in addition, the distance between the corresponding water molecules is less than 1.8 Å when the two structures are superposed. The 30 water molecules remained invariant with respect to all the ten possible pairs involving the five structures. The positions of these 30 water molecules in the low-humidity monoclinic lysozyme (Madhusudan *et al.*, 1993) and the interaction and the distance criteria mentioned above, were used to check their presence in monoclinic lysozyme. 21 and 15 of these water molecules are present in A1I and B1I, respectively; these numbers increase to 24 and 22 in A1F and B1F. The corresponding numbers in A2I and B2I are 13 and 21, respectively; they increase to 22 and 24 in A2F and B2F. The fact that the 30 water molecules remain 'invariant' with respect to the five well refined crystal structures suggest that many of them are likely to have constant positions with respect to the lysozyme molecule. The presence of larger numbers of them in the final models than in the initial models indicates the higher reliability of the final models. It is also interesting to note that the number of water molecules invariant with respect to A and B increases from 31 in 1I to 42 in 1F; the number increases from 19 to 46 when going from 2I to 2F. This again is a probable indication of the higher reliability of the final models.

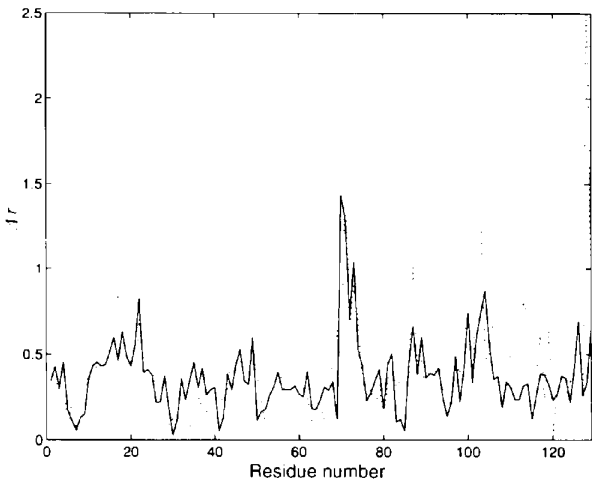

Fig. 4. Deviations of C^{α} positions in the molecule in the low-humidity form from those in molecule A (solid line) and molecule B (broken line).

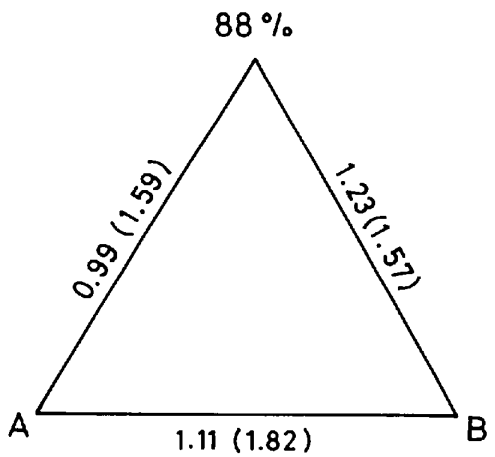

Fig. 5. R.m.s. deviations (Å) in atomic positions among molecules A and B, and the molecule in the 88% relative humidity form. The values in the parentheses result when the partially refined native structure (Rao *et al.*, 1983) is used.

All the crystallographic and the structural indices indicate that model 1F and 2F are reliable to nearly the same extent. Only the former is used in further discussion. The Ramachandran plot corresponding to this model shown in Fig. 2, demonstrates the geometrical acceptability of the model.

3.2. *Comparison with the low-humidity form*

The overall conformation of the two crystallographically independent molecules in the structure is very similar to that observed in other crystal forms of lysozyme. It is, however, of interest to compare the geometries of the two molecules between them and also with that observed in the low-humidity form ($P2_1$; $a = 26.90$, $b = 58.95$, $c = 31.33$ Å, $\beta = 111.9°$) which, as mentioned earlier, contains only one crystallographically independent protein molecule and has a low solvent content (Madhusudan *et al.*, 1993). A superposition of C^{α} atoms of the three molecules is illustrated in Fig. 3, while the deviations of these atoms in the low-humidity form from those in molecules A and B are shown in Fig. 4. Fig. 5 shows the r.m.s. deviations in atomic positions among the molecules

after superposition. The r.m.s. deviation in atomic positions between the molecules *A* and *B* is lower than that in the incompletely refined structure (Rao *et al.*, 1983). The same is true about the deviation between the molecules *A* and *B* on the one hand and the low-humidity form on the other (Madhusudan *et al.*, 1993). Thus, not surprisingly, random errors in structures tend to systematically overestimate differences between them; the differences are reduced with refinement.

A total of 232 main-chain atoms belonging to the two molecules in the native structure deviate by 0.5 Å or more when they are superposed individually on the molecule in the low-humidity form. Of these, 14 belong to the rigid regions, 112 to the moderately flexible regions and 106 to the highly flexible regions of the molecule. They account for 4.1, 21.9 and 59.6% of the three regions, respectively. The differences between the main-chain atoms in molecules *A* and *B* in the native structure also follow a similar distribution. Thus, again not surprisingly, the flexible regions are more susceptible to changes brought about by water-mediated transformations, as indeed by environmental differences. This is the reason why water-mediated transformations could be used as a tool for delineating the rigid and the flexible regions of the molecule (Madhusudan & Vijayan, 1991; Radhakishan *et al.*, 1995).

It is also of interest to enquire if the nature of atomic movements during the water-mediated transformation is the same in the two crystallographically independent molecules. To do so, molecules *A* and *B* were superposed on the molecule in the low-humidity form. Then, for each residue the angle subtended by the C^{α} of molecule *A* and that of molecule *B* on the C^{α} of the molecule in the low-humidity form was calculated. As a rough indication, the movement of C^{α} in the two molecules during the transformation is nearly in the same direction if the angle is acute and not if it is obtuse. It turns out that the angle is acute in 110 out of the 129 residues. Thus, it would appear that the nature of atomic movements during transformation is intrinsic to the protein molecule.

As in our earlier studies (Kodandapani *et al.*, 1990; Madhusudan & Vijayan, 1991; Radhakishan *et al.*, 1995) water molecules which are within 3.6 Å of one or more from protein O or N atoms are considered to constitute the hydration shell of the protein molecule. The hydration shells of the molecules *A* and *B* contain 125 and 131 water molecules, respectively. Of these 54 each are found in the hydration shell in the low-humidity form (Madhusudan *et al.*, 1993), using the criterion of invariance mentioned earlier. Thus, only less than half the water molecules in the hydration shell remain invariant during water-mediated transformation in monoclinic lysozyme while about two-thirds of the molecules remain invariant in the transformation in tetragonal lysozyme (Kodandapani *et al.*, 1990). This is presumably a reflection of the larger loss of water and hence the more drastic structural reorganization during the transformation in monoclinic lysozyme than during that in tetragonal lysozyme.

3.3. *Hydration and enzyme action*

It has been pointed out earlier (Madhusudan & Vijayan, 1991) that as far as the main-chain atoms are concerned the 16 residues implicated in substrate binding, namely, Phe34, Glu35, Asn37, Asn44, Asn46, Asp52, Gln57, Asn59, Trp62, Trp63, Asp101, Asn103, Ala107, Trp108, Val109, Arg114 (Imoto, Johnson, North, Phillips & Rupley, 1972; Ford,

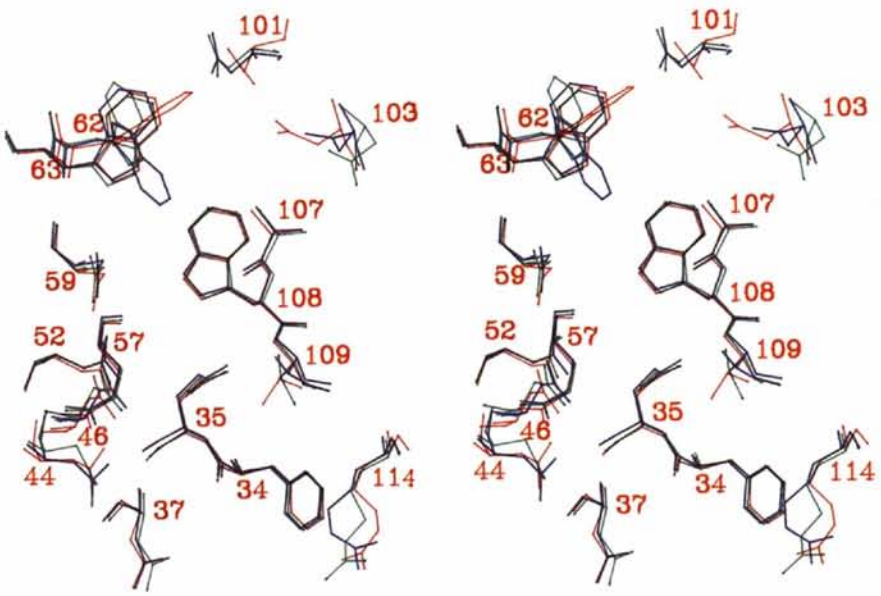

Fig. 6. Stereoview of the superposition of the active-site residues in the low-humidity and the native structures. The colour code is same as used in Fig. 3.

Johnson, Machin & Phillips, 1974; Perkins, Johnson, Machin & Phillips, 1978; Kelly, Sielecki, Sykes, James & Phillips, 1979), are no more flexible than the rest of the molecule. The deviations in these atoms among molecules *A* and *B* in the native structure and that in the low-humidity form corroborate this observation. As illustrated in Fig. 6, the situation is different in the case of the side chains of these residues. They show markedly higher flexibility than those in the other regions of the molecule. Altogether 241 side-chain atoms in the two molecules deviate by 1 Å or more from their positions in the molecule in the low-humidity form. Of these as many as 46 belong to the 32 active-site residues (out of a total of 258) in the two molecules. The deviation is most marked in Trp62, the side chain of which is known to invariably move during inhibitor binding. Thus, the structure further emphasizes the relationship between mobility, hydration and enzyme action (Kodandapani *et al.*, 1990; Radhakishan *et al.*, 1995).

Only one well refined structure of a lysozyme inhibitor complex is available in the Protein Data Bank (Cheetam, Artymuik & Phillips, 1992). The C^{α} positions in this complex and those in the low-humidity form were separately superposed on those in molecule *A* and those in molecule *B* (Fig. 7). Using the indication outlined earlier (when comparing atomic movements of molecules *A* and *B* during water-mediated transformation), the direction of displacement of C^{α} from molecule *A* to the complex and that from molecule *A* to the

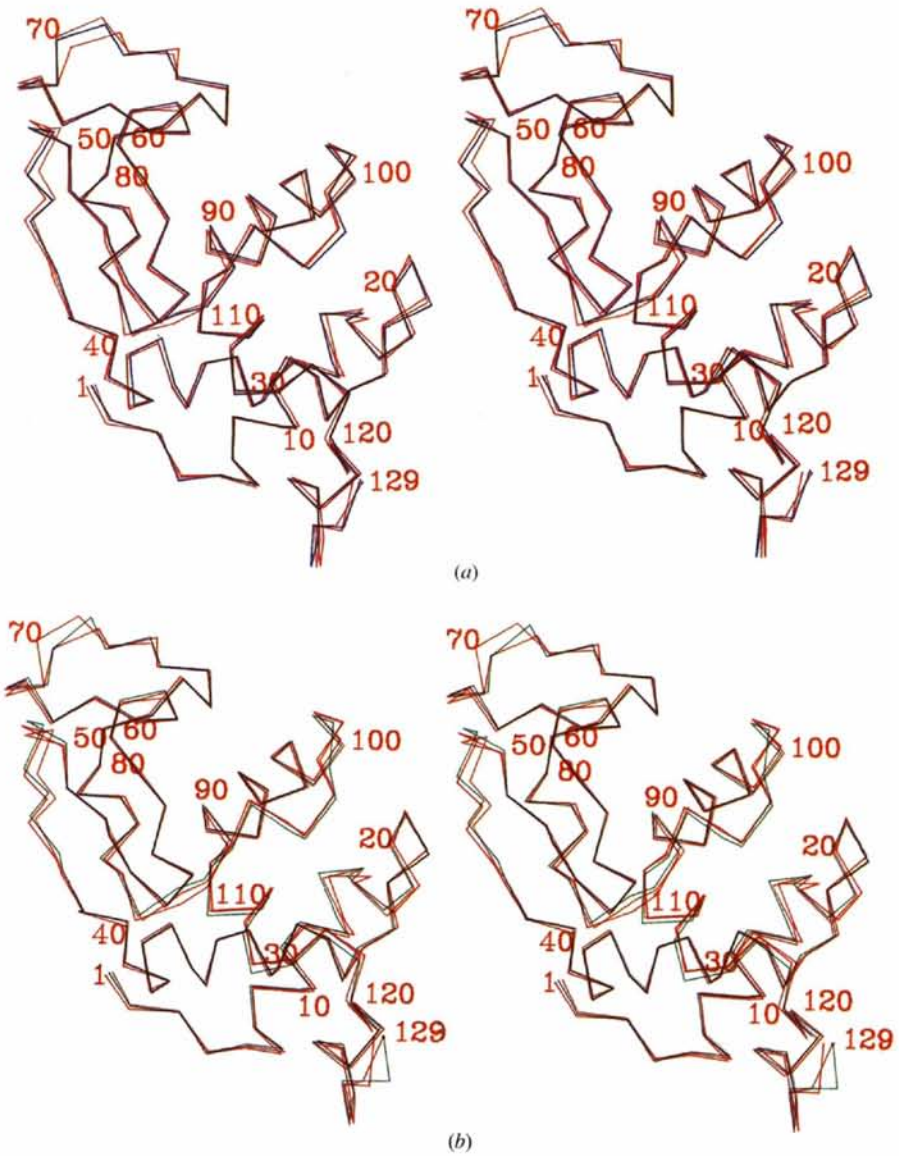

Fig. 7. Stereoview of the superposition of the C^{α} atoms in the low-humidity form and the inhibitor complex on those in (*a*) molecule *A* and (*b*) molecule *B*. The molecule in the inhibitor complex is in brown while the same colour code as in Fig. 3 is followed for others.

molecule in the low-humidity form is roughly the same in 104 of the 129 residues; the corresponding number in the case of molecule *B* is 121. Thus, the overall changes in the molecular geometry resulting from partial dehydration appear to be related to those which occur during catalysis, an observation similar to that made in the case of ribonuclease A (Radhakishan *et al.*, 1995).

4. Concluding remarks

The results presented here further indicate that the refined atomic coordinates could be in considerable error in the flexible regions of the protein molecule while they are likely to be more reliable in the rigid regions. When two independently refined models of the same structure are available, they can be reconciled to produce an improved model, starting from the regions of the molecules where the two models are in reasonable agreement. A comparison of the native monoclinic lysozyme structure with that of the 88% form demonstrates that the flexible regions of the molecule are highly susceptible to changes brought about by water-mediate transformation which thus provide a good tool to help delineate the rigid and flexible regions of the protein molecule. A comparison involving these structures and a lysozyme–inhibitor complex also emphasizes the relationship between mobility, hydration and enzyme action.*

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* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1UCO, R1UCOSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: SE0182).

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