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Macromolecular crystallography research at Trombay

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Abstract. Neutron diffraction studies of hydrogen positions in small molecules of biological interest at Trombay have provided valuable information that has been used in protein and enzyme structure model-building and in developing hydrogen bond potential functions. The new R-5 reactor is expected to provide higher neutron fluxes and also make possible small-angle neutron scattering studies of large biomolecules and bio-aggregates. In the last few years infrastructure facilities have also been established for macromolecular x-ray crystallography research. Meanwhile, the refinement of carbonic hydrases' and lysozyme structures have been carried out and interesting results obtained on protein dynamics and structure-function relationships. Some interesting presynaptic toxin phospholipases have also been taken up for study.

Keywords. Protein crystallography; enzyme mechanism; neutron diffraction; x-ray diffraction; carbonic anhydrase; lysozyme; presynaptic toxins.

1. Introduction

The function of biological macromolecules is often dependent on the ability of these molecules to transfer protons efficiently, which invariably involves active site hydrogen bonding. The advent of neutron diffraction has enabled the accurate determination of hydrogen atom positions in small molecules of biological interest like amino acids and peptides and the stereochemistry of the hydrogen bonds in these structures (Ramanadham and Chidambaram 1978). These studies have provided valuable information for use in protein and enzyme structure model-building and have also enabled the development of potential functions for hydrogen bonds between peptide groups (Chidambaram *et al* 1970) to be used in protein conformation studies. Neutron diffraction of biological molecules have been studied at Trombay using the neutron beams from the CIRUS reactor and a computer-controlled neutron diffractometer (Momin *et al* 1978). The latter and an x-ray diffractometer based on the same design are incidentally the first (and so far the only) computer-controlled diffractometers built in the country. The commissioning in 1984 of the 100-MW research reactor R-5 which will have several times the neutron flux of the CIRUS reactor will facilitate study of biological molecules using neutron beams. This reactor with a built-in cold source will also have a facility for small-angle neutron scattering (K R Rao, Private Communication) that can be used for studying large biomolecules and bio-aggregates using the powerful contrast-matching method with deuterium substitution.

X-ray diffraction techniques are most suited for studying globular protein and enzyme structures though some neutron diffraction studies have also been attempted at Brookhaven and Grenoble (Chidambaram 1981). The study of conformation of proteins was initiated by Prof. G N Ramachandran in India and he and his colleagues have made outstanding contributions which are too well-known to be recounted here.

The need for experimental studies in India of macromolecular structures especially protein structures, by x-ray diffraction methods has been felt for sometime. In 1978, when there was little effort being made in India in this direction, we decided to initiate a programme of study of enzyme structure and function by x-ray diffraction methods. From the instrumentation and computational knowhow generated from our earlier crystallography work and the wide interest in related fields in other divisions in BARC working on the biochemistry aspects of proteins and other macromolecules, our group seemed particularly well-suited to undertake this research.

1.1 *Why protein structure?*

Proteins are one of the most important components of any living system and carry out all the vital catalytic functions of the organisms. Protein enzymes exhibit a remarkable specificity towards their substrate. This specificity of the enzyme is mostly due to the spatial geometry of the active site of the enzyme, which is determined by the three-dimensional structure of the enzyme itself. Thus an understanding of the molecular aspect of enzyme catalysis necessarily requires the understanding of the three-dimensional structure of the enzyme. The elucidation of the enzyme mechanism is a difficult process and requires the study of the three-dimensional structure of the enzyme at different pH values and also of the enzyme-inhibitor and the enzyme-substrate complex (Blake *et al* 1967; Lipscomb *et al* 1968; Blow *et al* 1969; Kannan *et al* 1977b). The determination of the active site geometry requires the availability of accurate coordinates of the atoms that make up the protein structure. The structure of the solvent molecules that are important components of the *live* enzyme has also to be determined with a good degree of precision. A knowledge of the mode of inhibitor binding is also essential for an understanding of drug interaction with proteins (Kannan *et al* 1977c).

2. Protein structure determination

The all-important tertiary structure of the protein can be determined only by single crystal x-ray diffraction analysis. The method of isomorphous replacement, *i.e.* use of different heavy atom derivatives of the protein, has proved to be the single most important method to elucidate the structure of proteins (Green *et al* 1954; Blundell and Johnson 1976). Where complete or partial structure of a related protein is available, the application of the molecular replacement methods, *i.e.* the use of rotation and translation function (Rossmann 1972), may be fruitful in arriving at the structure.

The application of either the method of isomorphous replacement or the molecular replacement method results in an electron density map calculated from

$$\rho(x, y, z) = \frac{1}{V}\sum_h\sum_k\sum_l |F_0(hkl)| \exp i[2\pi(hx + ky + lz) - \alpha(hkl)], \tag{1}$$

where $|F(hkl)|$ is the structure amplitude of the reflexion (hkl) and $\alpha(hkl)$ is the phase angle calculated by the methods mentioned above. The electron density maps thus obtained are then interpreted either in an optical comparator (Richards 1968) or in a graphics display (Jones 1978) and the structure of the protein built up. This is of course a very critical operation and the success of the interpretation depends on the quality of the electron density maps and the availability of atleast partial amino acid sequence of the protein (Kannan 1977). The quality of the electron density map is dependent on the

quality of the diffraction data collected, *i.e.* the accuracy of the measurement of the structure amplitude for the native protein and the heavy atom derivatives. The quality of the map is dependent also on the quality of the phases $\alpha(hkl)$. These are entirely dependent on the quality of and the number of heavy atom derivatives used in the calculation in the case of the isomorphous method and on the degree of structural homology between the known and unknown structure in the case of the molecular replacement method.

It is important to stress that the quality of the crystals used and their stability to x-ray radiation are primary requisites to a successful solution of the protein structure. Thus crystallization of the native protein and the heavy atom derivatives assumes added significance as these are by no means well-established techniques like the rest of the protein structure determination.

The structure once determined as outlined above forms the basis for a more thorough analysis. The coordinates obtained are subjected to crystallographic refinement by a combination of least squares methods, and most important of all, inspection and interpretation of difference electron density maps where the amplitude $|F(hkl)|$ is now either $(|F_{obs}(hkl)| - |F_{cal}(hkl)|)$ or $(|2F_{obs}(hkl)| - |F_{cal}(hkl)|)$. During refinement the solvent molecules are also interpreted and their coordinates incorporated to get a complete tertiary structure. It must be stressed here that without locating the solvent molecules which are part of the protein structure, the determination of the tertiary structure of a protein is incomplete. Another important purpose of the refinement is to identify and include those amino acid residues which were not interpretable or wrongly identified during the course of the interpretation of the isomorphous or molecular replacement electron density maps.

A study of a protein structure is in itself very rewarding. However, the use of the structure results towards understanding functions of the protein is still more important and should be a natural goal for the protein crystallographer. This requires the availability of the accurate native protein structure at different pH values and also the accurate structure of the protein-substrate and protein-inhibitor complexes. Thus it is always necessary to obtain the structure of the protein under these varying conditions, a not so trivial task in terms of data collection and computation and electron density map inspection and not least the assimilation and interpretation of the biochemical-biophysical and enzyme kinetic data with reference to the structure of the protein in the different environments. It is thus apparent that a successful macromolecular work requires a well organised infrastructure facility in terms of powerful x-ray diffraction equipment, a well-equipped biochemical laboratory facility, automatic diffractometry or film recording equipment, automatic film measuring facility and not least of all good high speed computing facility and associated computer programs for doing the varied calculations needed for protein structure-function-inhibition analysis.

In the subsequent sections we will discuss the infrastructural facility at Trombay we have set up from 1979 onwards and also the results of the macromolecular analysis we have undertaken here.

3. Infrastructure at Trombay

3.1 *Instrumentation and laboratory facilities*

The protein crystallography group has a modest biochemical facility for the rudimentary but necessary protein purification work and has also access to the

instrumentation at the Biochemistry Division of the Bhabha Atomic Research Centre. The equipment set up by the group consists of a locally manufactured cold cabinet for maintaining the temperature of samples during preparation at 4°C, an automatic fraction collector designed and fabricated by the Central Workshops and the Reactor Control Division, BARC (Koppikar *et al* Personal communication), apart from other essential instruments like pH meters, gel electrophoresis apparatus and a binocular wide field microscope for crystal viewing during crystallization and also for crystal mounting for x-ray diffraction work. Many of the crystallization apparatuses (Blundell and Johnson 1976) like zeppezaur capillaries, vapour diffusion chambers for normal mode and hanging drop mode have also been made by the group. The group has successfully crystallized proteins and reported the first ever protein crystallization and crystal data in India using these and other facilities like dialysis and solution interfacing in melting point capillaries. Snake venom toxin proteins Notexin and Notechis II-5 have been crystallized at Trombay and are currently under investigation by x-ray crystallographic methods (Kannan *et al* 1981).

The x-ray diffraction equipment consists of a stabilized x-ray generator designed and fabricated by the scientists at BARC, Bombay and VEC, Calcutta and a high brilliance rotating anode (the latter is also used for energy-dispersive high-pressure diffraction research). The VEC-BARC generator uses the Phillips fine focus x-ray tubes with copper anode. For photographic data collection, there is a precession camera and an Arndt-Wonacott oscillation camera. It is also being proposed to modify an indigenous microprocessor-controlled x-ray diffractometer design to suit the crowded reciprocal lattices of macromolecular crystals.

The x-ray diffraction films are processed on a computer-controlled two-dimensional microdensitometer, which is also suitable for processing radiographs and other images on films. The map interpretation is done on a three-dimensional graphics display system available with the Computer Section at BARC.The group uses mostly the Prime 450 with the Computer Section at BARC and the VAX 11/780 at TIFR for their current computational needs.

3.2 *Computer programs*

Macromolecular structure determination and refinement requires a large number of computer codes. Often the programs used by the group have been available on computers other than those accessible to them. This has necessitated the conversion of a large number of computer codes for the protein group's day-to-day needs. A major programming effort has been that for the online control of the microdensitometer by the PDP 11/34 computer under the RSX11M operating system. The group has written the driver routine (Pal *et al* 1983) for the control and also converted Allan Wonacott's film processing programs written originally for a Nova computer system to the PDP 11/34 system under the RSX11M operating system using the driver developed at BARC. These include programs to index still photographs, processing of precesion, oscillation photographs and absorption streak photographs (Pal *et al* 1983) and programs for film scaling and Lorentz and polarization corrections.

The data obtained from the data processing programs above are being handled by the Protein program package kindly made available to us by Dr. Steigeman, Max Planck Institute for Biochemistry, Munich, Germany for the VAX 11/780 computer. This also includes, among other things, the structure factor and electron density

calculation programs. This program also forms the essential interface to the map manipulation programs so essential to use on the graphics system. The maps thus prepared are transmitted to the PDP 11/34 through a computer link from the Prime 450 computer, the software for which has been written at BARC (R S Mundada and R K Talwar, Private Communication) for interpretation on the graphics with the FRODO programme package (Jones 1978).

The refinement of the heavy atom derivatives and the calculation of the isomorphous phases are possible through a number of programs available to the group (Jarup *et al* 1970) and also on the VAX 11/780 computer with the Munich program system.

The protein crystallography group has made effective use of the restrained least squares (Hendrickson and Konnert 1980) and the constrained-restrained least squares (CORELS) (Sussman *et al* 1977) methods to refine the protein structures described below (Kannan and Ramanadham 1981; Ramanadham *et al* 1980, 1981; Ramanadham 1982). The program systems used for this purpose have been implemented on several computers. All the above programs have been used extensively to refine the human carbonic anhydrase B and triclinic hen egg white lysozyme structures. Interpretation of the electron density maps also requires a complete set of contour maps drawn on transparent plastic sheets, as a number of sections of the unit cell are stacked together in a suitable way. This is done by the program PLUTO which also gives the molecular skeleton plotted and superposed on the contour maps. This program is operational on Prime 450. A fast fourier transform (FFT) program to calculate the electron density sections which is input to PLUTO is also operational on Prime 450.

For the use of the molecular replacement method the fast rotation function program of Crowther (Rossmann 1972), the slower rotation function program of Rossmann (1972) as also the translation function program are available. Stereo-drawings of the molecular model or selected parts of the molecule are obtainable by the use of the program ORTEP2.

4. Structure refinement and function of proteins

We give below a summary of the protein crystallographic studies carried out at Trombay at the same time as the necessary infrastructure facilities for experimental macromolecular crystallography research were being built up.

4.1 *Presynaptic toxins*

A programme of structure-function studies on presynaptic toxin phospholipases has been initiated at Trombay. The presynaptic toxins are widely distributed in nature and are present in good quantities in the venom of some snakes. They are broadly classified into monomeric toxins and multimeric toxins. They also possess phospholipase A2 activity and the amino acid sequence shows very large homology with snake venom, bovine and porcine phospholipases. The Australian tiger snake (Notechis Scutalus Scutalus) is a rich source of the presynaptic toxins of the monomeric type. There are a number of fractions (Halpert and Eaker 1976) which show toxicity and phospholipase activity and also some which have only sequence homology but no toxicity or phospholipase activity. Three of these proteins are under investigation at Trombay. They are Notechis II-4 or Notexin, Notechis II-1 and Notechis II-5. Of these Notechis II-1 shows no toxicity and Notexin is more toxic than Notechis II-5. These toxins are

basic proteins with 119 amino acid residues cross-linked by several disulphide bridges. Notechis II-5 was the first protein to be crystallised and characterised in India and was reported by Kannan *et al* (1981). The homology with porcine and bovine phospholipases will be used and the structure solution attempted by molecular replacement methods. It may be possible to arrive at the toxicity centre of these toxins by comparing their three-dimensional structures which will help in finding the mechanism of presynaptic toxin action. The unit cell dimensions of these two toxins (Kannan *et al* 1977a) are

Notexin : $a = b = 75{\cdot}03$ Å, $c = 49{\cdot}04$ Å, $\alpha = \beta = 90°$ and $\gamma = 120°$. 6 molecules in a trigonal unit cell with space group $P3_121$ or $P3_221$

Notechin II-5 : $a = 146$ Å, $b = 43{\cdot}5$ Å and $c = 39{\cdot}0$ Å, $\alpha = \beta = \gamma = 90°$. 8 molecules in an orthorhombic unit cell with space group $P2_12_12_1$.

4.2 *Lysozyme*

Lysozyme was the first enzyme and the second protein whose structure was resolved at high resolution by x-ray diffraction technique (Blake *et al* 1965). The enzyme lyses polysaccharide-like polymers of N acetyl glucosamine and N-acetyl muramic acid.

Lysozyme is a monomer of 129 amino acids of molecular weight 14 600. There are four disulphide bridges. Hen egg white lysozyme has been crystallised in a number of crystallographic forms. Blake *et al* (1965) investigated the structure of the tetragonal form of the enzyme crystals. Jensen and colleagues (Ramanadham *et al* 1980) have studied the triclinic modification for which x-ray diffraction data extends to about 1 Å resolution. The initial structure in the triclinic unit cell was derived by the application of molecular replacement methods and subsequently the structure was refined by difference fourier methods (Ramanadham *et al* 1980). The structure was then refined by Ramanadham *et al* (1981) by restrained least squares method.

The restrained least squares refinement of triclinic hen egg-white lysozyme at 2 Å resolution was initiated and carried out to an *R*-factor of 0·179 at the University of Washington, Seattle, by Jensen and colleagues (Ramanadham *et al* 1980). This work has been continued at Trombay in collaboration with the Seattle group. The present *R*-factor is 0·169.

The restrained least squares method utilizes the known geometry of the different protein groups and introduces various geometrical restraints in the least squares procedure as a set of additional observations, thus improving the overdeterminacy ratio.

The quantity minimised is

$$\Phi = \Phi_1 + \Phi_2 + \Phi_3 + \Phi_4 + \ldots, \tag{2}$$

where Φ_1 is the conventional structure factor term in the least squares procedure.

$$\Phi_1 = \sum (|Fo| - |Fc|)^2,$$

Φ_2, Φ_3, etc. are terms for the different restraints for planar groups, atomic distances, chiral volumes, non-bonded contacts, isotropic thermal parameters, conformational parameters, symmetry features and so on (Hendrickson and Konnert 1980).

In the refinement of Lysozyme (Ramanadham *et al* 1981) 4961 parameters were adjusted by the restrained least squares procedure. There were 1001 protein and 239 solvent atoms and one scale factor as against 7131 independent Fobs structure

amplitude values within 2 Å resolution and about 3000 restraints. The root mean square deviation in the distances between different atoms from ideal values were less than 0·05 Å for the final model.

Protein crystals suffer from static and dynamic disorder and it would be difficult to assign a meaning to the temperature factor in a conventional sense. However it has been noticed that the temperature factor of the protein atoms in triclinic lysozyme was very low compared to many other protein structures. The average B value for the molecule is about 8·0 $Å^2$, much smaller than in tetragonal lysozyme and this seems to be related to a larger fraction of the water molecules being ordered in this structure (Ramanadham *et al* 1981).

Mn^{2+}, Co^{2+} and Gd^{3+} have been found to bind to the active site of Lysozyme (Kurachi *et al* 1975 and Teichberg *et al* 1974). It has also been observed that divalent copper ions inhibit lysozyme. The binding of Cu^{2+} to tetragonal lysozyme has been reported by Teichberg *et al* (1974), Ramanadham *et al* (1981) have studied the binding of Cu^{2+} to triclinic lysozyme at Trombay using the 2 Å data collected at University of Washington. The structure of the Cu^{2+} lysozyme complex was refined by restrained least squares method using the refined coordinates of the triclinic lysozyme structure to a crystallographic *R*-factor of 16·9 % after two cycles of refinement. Ramanadham *et al* (personal communication) have not observed any significant deviations of the protein atoms on copper binding. They had observed that one Cu^{2+} ion was found in the vicinity of Glu 35 in the active site and a second copper ion was bound to the enzyme in the vicinity of His 15 residue.

4.3 *Human carbonic anhydrase*

Mammalian erythrocytes are a rich source of carbonic anhydrase enzyme which reversibly catalyses the interconversion of bicarbonate to carbon dioxide and water. The human erythrocytes contain, apart from the major component haemoglobin, two carbonic anhydrase isoenzymes, form B (HCAB) and form C (HCAC). The two isoenzymes are homologous to each other with about 260 amino acid residues and an essential Zn^{2+} ion and have a molecular weight of 30000. There are no disulphide bridges in these enzymes. The isoenzymes differ in their catalytic efficiency. Aromatic sulphonamides which are specific inhibitors of these enzymes also exhibit differing inhibitory properties. The enzymes, however, are highly efficient in the catalysis of CO_2 bicarbonate interconversion with a turnover rate of 10^6 moles of CO_2 per mole of enzyme per sec. The mechanism of action of these fascinating enzymes are still hotly debated by the different workers in the field (Lindskog *et al* 1971).

The three-dimensional structure of HCAB (Kannan *et al* 1975) and HCAC have been now known for some time (figure 1) (Kannan *et al* 1971 and Liljas *et al* 1972). Kannan *et al* (1981) have been refining the structure of HCAB at 2 Å resolution to get the complete tertiary structure of the enzyme including the solvent structure in the crystals. The solvent structure may play an important role in the mechanism of action of the enzyme. The enzymes also show a different folding pattern compared to many of the other protein structures known. The molecule is twice as large as lysozyme discussed above. The diffraction data had been collected at Uppsala, Sweden on photographic films using precession cameras (Kannan *et al* 1975) during the course of the high resolution structure determination. The coordinates of HCAB had been measured from a Kendrew Watson skeletal model and the model adjusted to conform to stereochemical standards

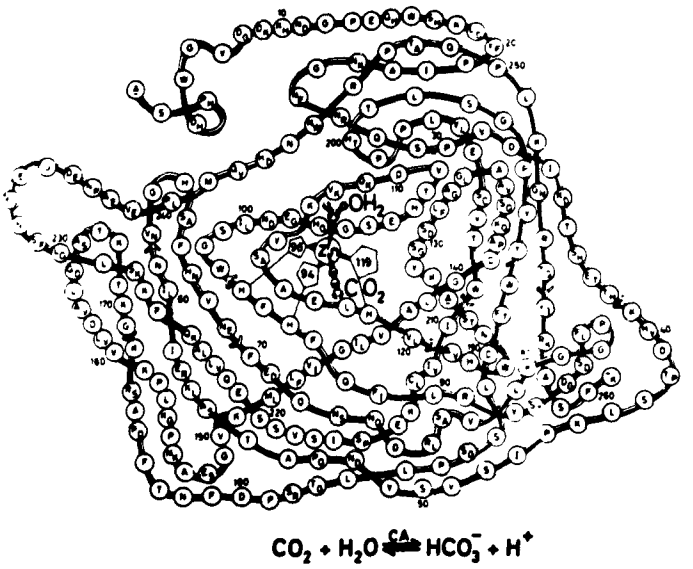

Figure 1. Schematic diagram of the three-dimensional structure of carbonic anhydrase molecule with the amino acid sequence of the HCAB and HCAC in the one letter code.

using the model building program (Diamond 1971). The crystallographic *R*-factor was about 50%. The model had been fitted to the isomorphous map in a 3D graphics display by one of us at Max Planck Institute for Biochemistry, Munich, Germany using the programme FRODO (Jones 1978) and the resultant model had been refined with the real space refinement program (Diamond 1974) on a Siemens 4004 computer at Munich. The *R*-factor was 39·5% for the 14578 observed reflections in the 5 to 2 Å sphere. The weak unobserved reflections had been dropped at this stage.

The structure was then refined by Kannan and Ramanadham (1981) at Trombay using the restrained least squares programs, discussed earlier in the section on lysozyme, to 36·5% R-value for the 3 Å resolution data and later to 32·3% for the 14524 reflection in the 5 to 2 Å sphere where individual isotropic temperature factors were used in the refinement. However the improvements in *R*-factors after 8 cycles of least squares refinement tapered off. The model was now compared with the electron density maps and wrong orientation of the residues corrected. The three-dimensional graphics facility at the Wallenberg Laboratory, Uppsala, Sweden was used during a visit by one of us for this purpose in collaboration with Dr T A Jones (Kannan *et al* 1982). The model was refitted to the isomorphous electron density map in the graphics system. Kannan *et al* (1982) have reported that the model required refitting in a number of regions and also a number of side chains and even main chain atoms dropped from the refinement due to difficulties in fitting to the isomorphous electron density map. Of the total of 2200 protein atoms they had included only about 1850 atoms in the constrained-restrained least squares procedure COREL with one overall temperature factor. The *R*-factor had dropped after 2 cycles from 45·5% for the refitted model to 36% for the 6 to 2 Å data.

They then fitted the model to a ($|2F_o| - |F_c|$) electron density map in the graphics system. In this process a number of residues which were left out from the previous calculation were located and included in further refinement. In all they had done alternatively 4-model fitting sessions to the electron density maps on the interactive graphics system and 8 cycles of COREL S. At this stage only 4 residues at the amino terminus, some side chains especially lysines and all the solvent atoms were not included. The *R*-factor had dropped to 31 % with one overall temperature factor and to 26·7 % while refining two group temperature factors for every residue in the last two cycle (Kannan *et al* 1982).

The refinement has since been continued at Trombay. About 60 solvent molecules located within hydrogen bonding distance to relevant proteins groups from ($|F_o| - |F_c|$) electron density map were included and individual restrained isotropic temperature factors refined for all atoms using the restrained least squares procedure outlined in the section on lysozyme. After 2 cycles the *R*-factor had dropped to 22·7 %. The refinement using COREL S also gave an almost identical result giving a *R*-factor of 22·5 %. An inspection of the ($|F_o| - |F_c|$) electron density map resulted in 40 more solvent atoms being located, within hydrogen bonding distance to the relevant protein group or solvent atoms located in the earlier ($|F_o| - |F_c|$) map. The interactive three-dimensional graphics facility became available at this time at Trombay and the FRODO program (Jones 1978) system was made operational on the PDP 11/34 computer system connected to the graphics system by one of us. A ($|2F_o| - |F_c|$) map was then used to fit the refined coordinates and the solvent atoms (Kannan *et al*, personal communication). They have reported correcting the wrongly oriented side chains, especially the forked residues for a number of side chains. They also located another 60 solvent atoms from an inspection of the ($|2F_o| - |F_c|$) and the ($|F_o| - |F_c|$) maps. A number of side chain conformational angles were also corrected to near-staggered orientation in conformity with the electron density on the graphics system. It was observed by them that none of the least squares refinement programs was capable of correcting such errors and often forcing the residues to false minima. Thus only manual intervention could rectify such faults. After three more cycles of restrained least squares refinement the *R*-factor had dropped to 19·5 % (figure 2) for the reflections in the 5 to 2 Å sphere. All the 15524 reflection in the 10–2 Å sphere were then used by them, and a ($|2F_o| - |F_c|$) map and a ($|F_o| - |F_c|$) map computed and used on the graphics system for further improvement in the model-electron density fittings and solvent localization. Kannan *et al* (personal communication) were able to identify two more of the amino terminal residues, all but two lysine side chains and an arginine side chain as also about 150 solvent atoms either hydrogen-bonded to the protein or to the solvents, located earlier. Thus about 300 solvent atoms have been added to the protein structure so far.

They also have found some interesting solvent structure in the HCAB molecule. They found that the catalytically important Zn^{2+} has a solvent coordinated to it and is within hydrogen bonding distance to Thr 199. There is a solvent molecule bridging Glu 106 and Tyr 7 by hydrogen bonding to them. Glu 106 was also found hydrogen-bonded to Thr 199. A solvent hydrogen-bonded by Tyr 6 was also found hydrogen-bonded to His 64 located in the active site of the enzyme. Another interesting feature of the solvent structure seems to be their participation in breaking pleated sheet hydrogen bonding. A few such solvents have been located by Kannan *et al* (private communication).

Another important feature reported by Kannan *et al* is regarding the temperature factors. Internal residues and residues in the pleated sheet structure have very low

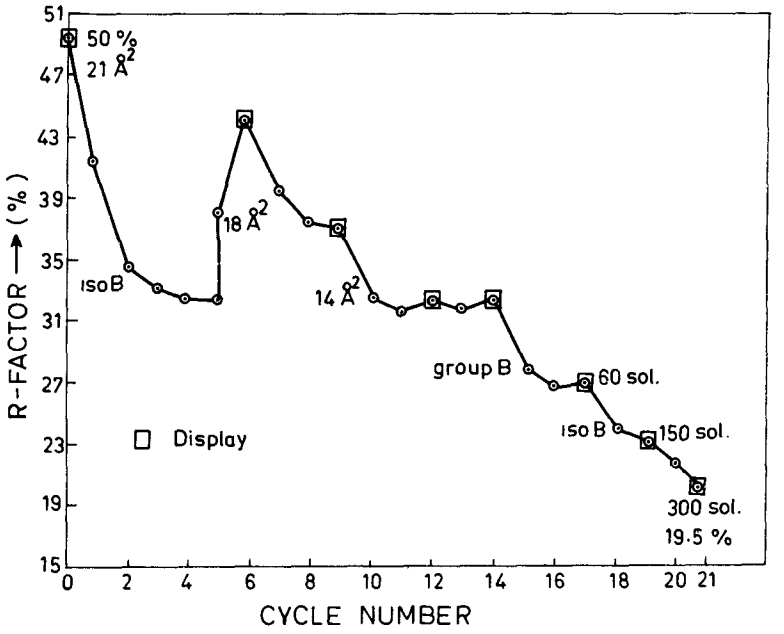

Figure 2. A plot of the descrepancy factor versus least squares cycle number in the refinement of HCAB. Reproduced with permission from Kannan *et al.*

temperature factor, in some instance 2·0 Å^2, and residues on the surface of the molecule, especially lysines, and other polar residues have very high temperature factors, sometimes as high as 30 Å^2. The solvents also have varying temperature factor from 6 Å^2 to 60 Å^2 depending on their location and hydrogen bonding. The average B-value for all the protein atoms is 12 Å^2.

The refined coordinates of HCAB has been used to calculate ($|2F_0| - |F_c|$) maps for a number of inhibitor complexes and one metal replacement for 2 Å^{2+} at Trombay. The results of these investigation agree well with those reported earlier by Kannan *et al* (1977b and 1977c) and Kannan (1980a and 1980b). The mechanistic proposal made by Kannan *et al* (1977b) would still be valid with minor modification in the light of the solvent structure found during the course of the refinement.

5. Conclusion

Neutron beam research at Trombay on small biomolecules will be enlarged in scope with the advent of the higher-flux *R*-5 reactor to include small angle neutron scattering studies of large biomolecules and bio-aggregates. We are now also fully equipped at Trombay to undertake macromolecular crystallography research with x-rays. The experimental facilities have been installed and a complete library of computer programs established. Meanwhile a number of protein structures have been refined and interesting results obtained on protein dynamics and structure-function relationships. We have also taken up the study of several new proteins.

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