# Form and function: X-rays in structural biology*

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**The most spectacular applications of X-ray diffraction during the last couple of decades have been concerned with biological macromolecules. Macromolecular crystallography has exquisitely demonstrated the relation between form and function at the molecular level. Its decisive influence pervades almost all aspects of modern biology and its applications. Happily, a viable and reasonably internationally competitive activity in this area has emerged in India during recent years. The effort at Bangalore, which forms an important component of this activity, encompasses lectins, viruses, hydration and plasticity of proteins, and enzymes.**

UNDOUBTEDLY the most important classes of biomolecules are nucleic acids and proteins. Nucleic acids are mainly, but by no means exclusively, informational molecules. Once synthesized using the information contained in nucleic acids, most of the rest of the work in living organisms is carried out by proteins, each of which is endowed with a characteristic three-dimensional form suited for its function. There are many other classes of biomolecules such as carbohydrates, lipids, etc. Structural biology is concerned with the structure, assembly, interaction and function of all these biomolecules. The structural complexity and functional variety of proteins are so extensive that the study of proteins is the most important component of structural biology followed by that on nucleic acids and other biomolecules, in that order. Most of the important biomolecules are polymeric macromolecules and the most important tool for investigating their structure has been X-ray crystallography. Indeed, a very substantial part of what we know of biomolecular structure and association has resulted from biological macromolecular crystallography.

## Historical development

Macromolecular crystallography began in 1934 when the X-ray diffraction pattern from the crystals of the digestive enzyme pepsin was recorded at Cambridge by J. D. Bernal and his student Dorothy Hodgkin (then Crowfoot)[1]. In the words of Dorothy Hodgkin and her associate D. P. Riley[2]:

'The history of the X-ray analysis of protein crystals began for many of us when the first X-ray diffraction photographs of single pepsin crystals were taken in 1934. The crystals were hexagonal bipyramids, 2 mm long or more, prepared by John Philpot while he was working for a short time at Uppsala. He had left his preparations in the refrigerator while he was off on a skiing holiday, and on his return was astonished to find how large his crystals had grown. He showed them to Glen Millikan, a visiting physiologist from California and Cambridge, who said, "I know a man in Cambridge who would give his eyes for those crystals". Philpot naturally offered him some crystals to take back in his coat pocket and so Millikan took them to J. D. Bernal'.

Soon after, young Dorothy returned to Oxford as an independent worker and there she photographed the X-ray diffraction pattern from the crystals of the protein hormone insulin[3], which she reckoned as the most exciting event in her life. Crystals of lactoglobulin were studied next[4]. In the meantime, work on haemoglobin and chymotrypsin was started at Cambridge by Bernal, Fankuchen and Perutz[5].

The thirties was a time when the solution of the structure of even a small molecule was considered to be a great intellectual achievement. As far as biology is concerned, even the exact chemical nature of proteins was not known. It is a tribute to the prescience and tenacity of visionaries like J. D. Bernal, Dorothy Hodgkin and Max Perutz that they thought of even attempting to investigate protein structure. Indeed, it took quarter of a century of sustained effort for the first definitive results on protein architecture to emerge.

In the meantime, X-ray fibre diffraction studies, pioneered by W. T. Astbury, began to yield exciting results. The X-ray diffraction pattern from a fibre contains only diffuse spots or streaks and it cannot be used to solve the structure *ab initio*. It however provides clues regarding the structure. Also, proposed models can be checked against fibre patterns. The most important discovery which relied on fibre data was undoubtedly that of the celebrated double-helical structure of DNA by Watson

*Dedicated to Prof. G. N. Ramachandran whose outstanding contributions have been a source of inspiration to us as we strive to approach the heights he reached a generation ago.

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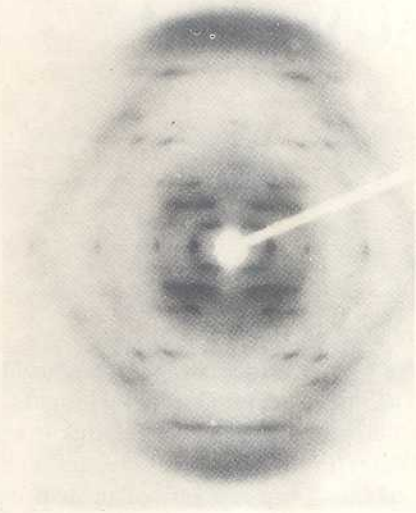

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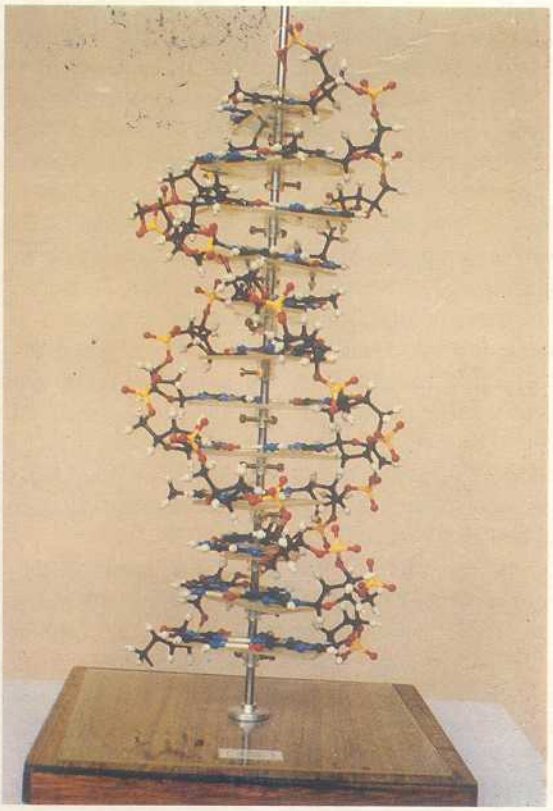

**Figure 1.** *a*, X-ray diffraction pattern from B-DNA. (Reproduced from the Ph D thesis of P. Parrack, Indian Institute of Science, Bangalore, 1988); *b*, A model of DNA. (Kindly made available by Manju Bansal.)

and Crick (Figure 1) (ref. 6), a structure which exquisitely demonstrates the relation between form and function. Yet another major contribution in the fifties based on fibre diffraction and modelling was the proposal of the triple-helical structure of collagen by G. N.

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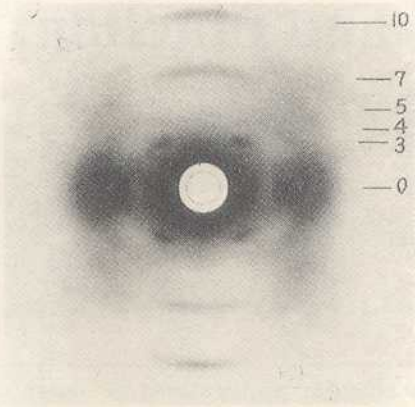


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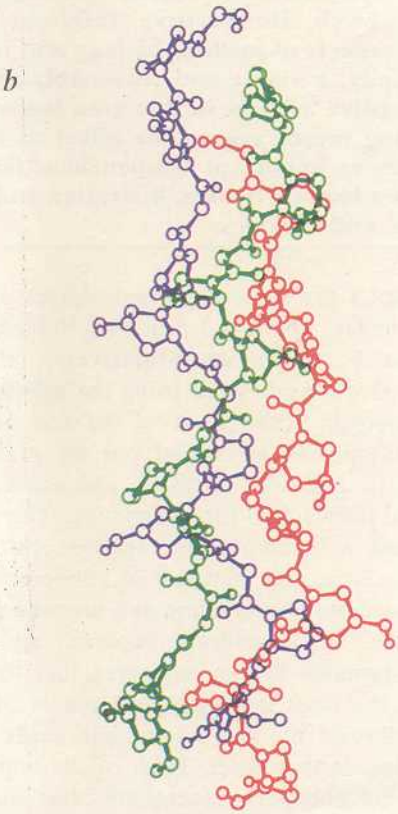

**Figure 2.** *a*, X-ray diffraction pattern from collagen. (Reproduced from ref. 95); *b*, Triple helical model of collagen. The three strands are coloured differently. Drawn using the coordinates provided in ref. 96.

Ramachandran and his post-doctoral associate G. Kartha (Figure 2) (refs 7, 8). The other spectacular success of modelling was the discovery of the secondary structural features such as α-helix and β-sheets by Linus Pauling[9,10].

In the middle of all this excitement, Max Perutz, John Kendrew and others were systematically progressing with the X-ray analysis of protein crystals. The first suc-

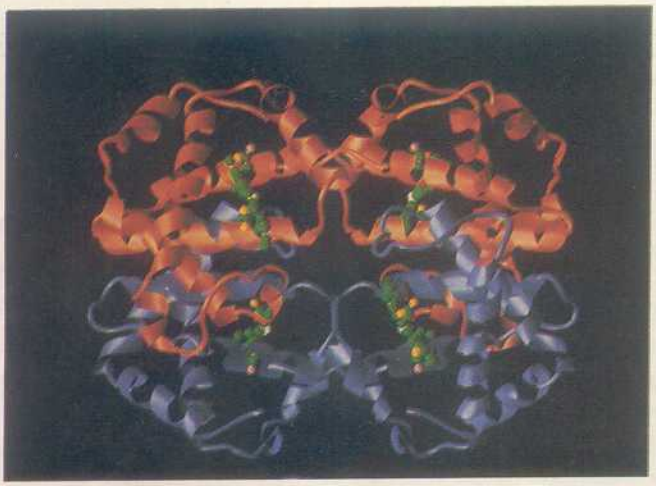

**Figure 3.** The structure of haemoglobin[97]. The α and the β chains are coloured differently. Atoms in the haem group are represented by spheres. This and the subsequent structural figures were prepared using the data taken from the Brookhaven Protein Data Bank[98]. This figure and figures 4, 5 and 7–11 were produced using RIBBONS[99].

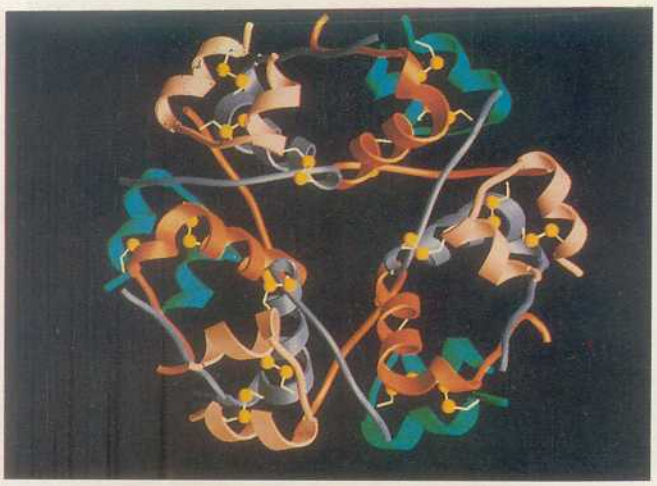

**Figure 5.** Hexameric 2Zn insulin (ref. 100). In each dimer, the monomers are coloured differently. So are the A and B chains in each monomer.

**Figure 4.** Lysozyme complexed with tetra-*N*-acetyl-chitotetrose. The tetrasaccharide is given in the ball and stick representation. (Unpublished results of K. Maenaka, M. Matsushima, H. Song, K. Watambe, and I. Kumagai.) Protein Data Bank reference number 11ZC.

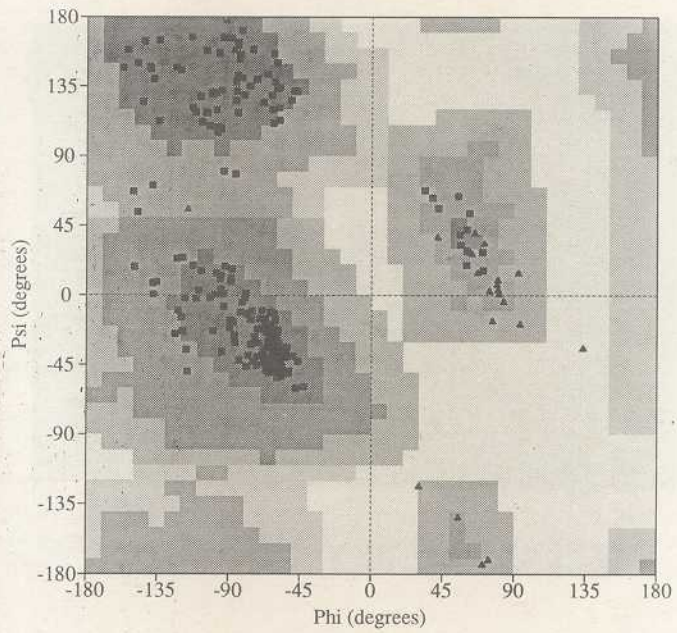


**Figure 6.** A typical Ramachandran plot (that of monoclonic lysozyme[72]) prepared using PROCHECK[101].

cess came in the late fifties and the early sixties when the structures of myoglobin and haemoglobin were solved[11,12]. Tetrameric haemoglobin (Figure 3), which is involved in the transport of oxygen and carbon dioxide, is among the most thoroughly studied proteins. Perutz and others have worked on haemoglobin in different states, from different sources and with different genetic defects. This monumental work provides a structural explanation for much of the chemistry, biology and pathology of haemoglobin. The first structure of an enzyme, that of lysozyme, was solved by David Phillips and his associates in the mid-sixties (Figure 4) (ref. 13). The structure for the first time demonstrated how an enzyme and its substrate fit each other like a lock and key. The structure of several other enzymes such as ribonuclease[14], chymotrypsin[15], carboxypeptidase[16], papain[17], and subtilisin[18], became available in quick succession. The area of biology which has been most directly enriched by protein crystallography from its

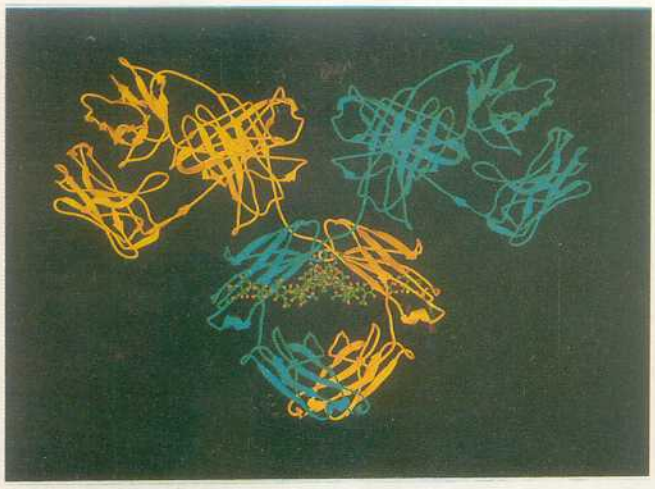

**Figure 7.** An immunoglobulin molecule[102]. The two chains are coloured differently. The covalently-linked carbohydrate is given in a ball and stick representation.

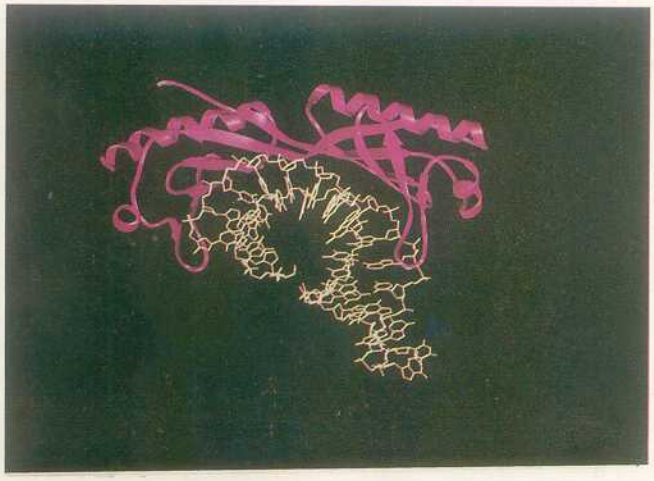

**Figure 8.** The TATA box binding protein complexed with DNA[25]. The skeletal model in white is DNA.

early days has indeed been enzymology. The first crystal structure of a protein hormone, that of insulin in the hexameric form (Figure 5) in which it exists in the pancreas, was also determined by Dorothy Hodgkin and her colleagues towards the end of the sixties[19]. During the preceding decades Dorothy Hodgkin worked on several other problems as well and solved the structures of such important molecules as cholesterol, penicillin and vitamin $B_{12}$, but insulin remained her first love and she persevered with it. In fact, those of us who were directly involved in the solution of the structure of insulin in 1969, namely, Guy Dodson, Eleanor Dodson, Tom Blundell and myself, were even not born when she first recorded the X-ray diffraction pattern of insulin in 1935!

Many of the stereochemical criteria that govern protein architecture were also developed in the sixties. The very well-known Ramachandran plot (Figure 6) (ref. 20) is the most important contribution in this area and it remains even today the most widely used means for representation and validation of protein structures.

## The global scenario

Macromolecular crystallography came of age by the late sixties and the early seventies even though the number of protein structures solved by the turn of the decade could be counted on one's fingers and the number of macromolecular crystallographers were perhaps around a hundred. Many more structures were solved in the seventies and most of the experimental and theoretical techniques were standardized. The next revolution in macromolecular crystallography started in the eighties and it still continues. The first and the most important technological advance that contributed to this revolution is perhaps the advent of position-sensitive detectors which made data collection and processing very much faster and more accurate than before even with an in-house conventional or rotating anode X-ray generator. Secondly, widespread accessibility to computer graphics made the interpretation of electron density maps and modelling fast, accurate and enjoyable. Thirdly, the increased computing power made it possible to very quickly try out different alternative approaches to structure solution and refinement. Finally, the availability of synchrotron radiation not only made it possible to collect data from weakly diffracting and very small crystals, but also opened up new areas such as anomalous scattering using tunable radiation[21] and kinetic crystallography[22].

The great strides in other areas of modern biology also decisively contributed to the revolution in macromolecular crystallography. Genetic engineering techniques made it possible to produce scarce proteins in large quantities. Techniques for the production of monoclonal antibodies and other immunological methods immensely helped structural studies of immunological systems. Site-specific mutagenesis and protein engineering made it possible to change the amino acid residues in a protein at will and to study the effects of these changes. Indeed, the close and organic interaction between biochemists and molecular biologists on the one hand and X-ray crystallographers on the other, has been responsible for many important advances in modern biology.

The crystal structures of hundreds of proteins and their assemblies are now available and the number is increasing rapidly. We now know a great deal about the fundamental principles of protein architecture and the

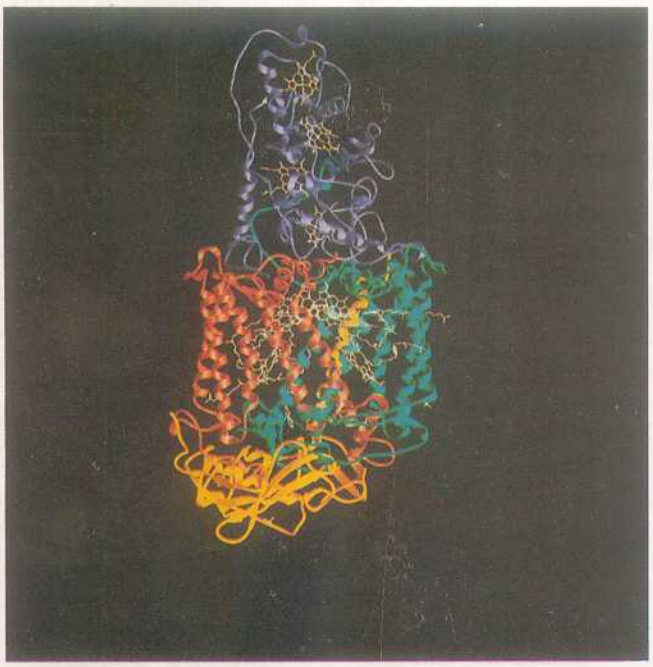

**Figure 9.** The photosynthetic reaction centre[27]. The different subunits are coloured differently. The skeletal models represent prosthetic groups.

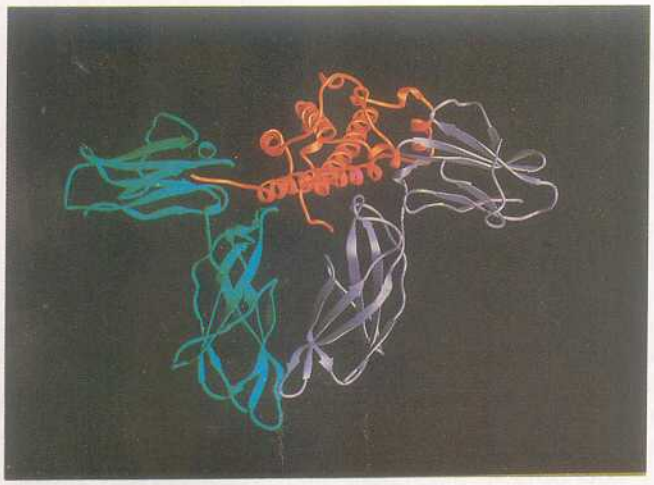

**Figure 10.** A complex of human growth hormone with the extracellular domain of its receptor[28]. The receptor is in green and violet while the hormone is in brown.

forces that stabilize it at different levels of organization such as the primary, secondary, tertiary and quaternary structures. We have learnt much about the anatomy and the taxonomy of protein structures. The influence of macromolecular crystallography pervades almost all areas of modern biology. Several families of proteins have been examined, and many evolutionary and structural relationship have been established. In almost all cases, structure illuminates the molecular basis of the function and also suggests further lines of investigation.

The area of influence of macromolecular crystallography is so vast that one cannot even begin to give representative examples. Perhaps enzymes, the biological catalysts, constitute the largest family of proteins with known structure. They encompass a variety of structures, sizes, shapes and mechanisms. Yet another area in which structural studies have made a major impact is immunology[23]. One of the early achievements in this area has been the elucidation of the detailed architecture of the antibody molecule (Figure 7). Great advances have been made during the last decade or more in unravelling the different facets of protein–nucleic acid interactions[24]. A recent example of such advances is the structure elucidation of a complex between the TATA box binding protein and DNA (Figure 8) (ref. 25) which is responsible for the initiation of transcription. Protein–carbohydrate interactions are also receiving considerable attention in recent years[26] and we shall touch upon this area in the context of the work at Bangalore. Biologically active multimolecular systems have recently been subjected to detailed X-ray analysis. Perhaps the best example of such a system is the photosynthetic reaction centre (Figure 9) (ref. 27). The recent determination of the structure of the human growth hormone receptor complex (Figure 10) also deserves special mention[28]. Another exciting area is concerned with the structure analysis of viruses[29], an area with an Indian presence. In addition, the current macromolecular crystallographic effort encompasses such important areas as protein engineering, drug design and protein folding.

The discussion so far has been concerned primarily with proteins. X-ray crystallography has played an important role in the structure elucidation of nucleic acids such as t-RNA[30]. Oligonucleotide crystallography led to the discovery of Z-DNA and the elaboration of the sequence-dependence of DNA conformation[31].

## The national scene

India has had a distinguished tradition in crystallography, a tradition that originated from C. V. Raman. India has also made a name for itself for contributions in the crystallography of small molecules. As indicated earlier, G. N. Ramachandran has been the world leader in the study of biomolecular conformation and the foundations of crystallography. Yet experimental macromolecular crystallography got off the ground in India only comparatively recently primarily for two reasons. First, the minimum critical financial inputs necessary for starting a protein crystallography programme was not available till the time in the early eighties when the Department of Science and Technology initiated its thrust area programmes. Secondly, interactions between crystallographers and biochemists, a necessary condition for a sustained biological macromolecular crystallography programme, were at a very low level even up to a couple

of decades ago. Furthermore, an element of diffidence in taking up in Indian conditions large, long-range projects, was also an inhibitory factor.

Serious efforts at initiating macromolecular crystallography in India started in the late seventies and the early eighties at the Molecular Biophysics Unit (MBU), Indian Institute of Science, Bangalore, and at the Neutron Physics Division, Bhabha Atomic Research Centre, Bombay. The efforts got a great impetus in the eighties due to the support from the Department of Science & Technology and through the UGC Centre of Advanced Study programme at MBU. In the meantime, the Bangalore centre was identified as a national nucleus for the development of macromolecular crystallography in the country and a modern Area Detector System was installed as a facility there. This facility and the interactions that took place around it did indeed make a great difference to macromolecular crystallography in India.

Until the early nineties, Bangalore and Bombay were the only centres where macromolecular crystallographic investigations were pursued. Recently active groups have emerged at the All India Institute of Medical Sciences, New Delhi; National Institute of Immunology, New Delhi; Saha Institute of Nuclear Physics, Calcutta; and the University of Madras. The other centres where work is expected to gather momentum soon include Bose Institute, Calcutta; Madurai Kamaraj University; National Chemical Laboratory, Pune; and Institute of Microbial Technology, Chandigarh. In addition to DST, the Department of Biotechnology has in recent years extended very considerable support to macromolecular crystallography. Furthermore, DBT's Bioinformatics programme has been of great help in terms of graphics and computational facilities. Other agencies like the Council of Scientific and Industrial Research and the Department of Atomic Energy have also shown increased interest in the area. With the support of DST and DBT, the facility at Bangalore has been upgraded and major new data collection facilities have been established at Bombay and Delhi. A similar facility is soon expected to be established at Calcutta so that macromolecular data collection facilities would exist in the southern, the western, the northern and the eastern regions of the country.

The scientific content of the efforts at Bangalore is outlined later. The work at BARC, Bombay, over the years has involved several projects. Undoubtedly, the most extensively studied protein at Bombay is carbonic anhydrase. K. K. Kannan and his colleagues have carried out detailed investigations on different forms of human carbonic anhydrase and their complexes with several sulphonamide drugs and other inhibitors[32–34]. Their contribution to the on-going efforts to explain the mechanism of action of this very efficient enzyme has indeed been considerable. Furthermore they have also solved the structure of the homologous buffalo enzyme[35]. Another system being studied by Kannan, M. V. Hosur and others is a multi-enzyme complex involving RUBISCO[36]. They have also determined the structure of gelonin, a ribosome inactivating protein[37,38]. M. Ramanadham and Hosur have been pursuing other projects with overseas collaboration[39,40]. The work at Bombay has also involved development of methodologies[41–43].

Although macromolecular work at the other centres started only recently, significant results have already begun to emerge from them. T. P. Singh at the All India Institute of Medical Sciences and his colleagues are involved in investigations on a double-headed proteinaceous inhibitor[44], and lactotransferin and lactoperoxidase from buffalo and camel[45,46]. They also have collaborative interactions with a German group on proteinase K–inhibitor complexes[47]. After carrying out some excellent modelling studies while setting up the X-ray laboratory[48–51], D. M. Salunke's group at the National Institute of Immunology is pursuing X-ray studies on an antibody against GnRH, several other immunologically relevant problems, a rat ribonuclease and barstar[52]. J. K. Dattagupta and his associates at the Saha Institute of Nuclear Physics have recently determined the structure of a chymotrypsin inhibitor from winged beans[53,54]. The macromolecular work of N. Gautam's group at Madras has been concerned with sequence-dependent and unusual DNA helices[55–57].

## The Bangalore effort

From modest beginnings, the macromolecular crystallography school at MBU has grown into a reasonably sized coherent group numbering about 25 including faculty members. The activity encompasses a number of long-range programmes spanning small proteins, multisubunit proteins and giant multi-molecular assemblies, and involves close collaboration with biochemists with similar interests. The problems addressed concern the structure, assembly, interactions, plasticity and hydration of proteins. As indicated earlier, the Bangalore centre has also functioned as a national nucleus for the development of macromolecular crystallography in the country.

An important long-range programme pursued by us in collaboration with A. Surolia and with the involvement of K. Suguna, is concerned with the structure and interactions of lectins. Lectins are multivalent carbohydrate-binding proteins and have attracted considerable attention on account of their ability to specifically bind to different cell surface carbohydrates which are the most important determinants of biological specificity. The major result to emerge from the lectin programme is the *de novo* structure determination, using the multiple isomorphous replacement method, of the tetrameric peanut lectin (Figure 11) (refs. 58–61), Mr 1,10,000, which specifically binds to the tumour-associated disaccharide called T-antigen. The structure, which took nearly a

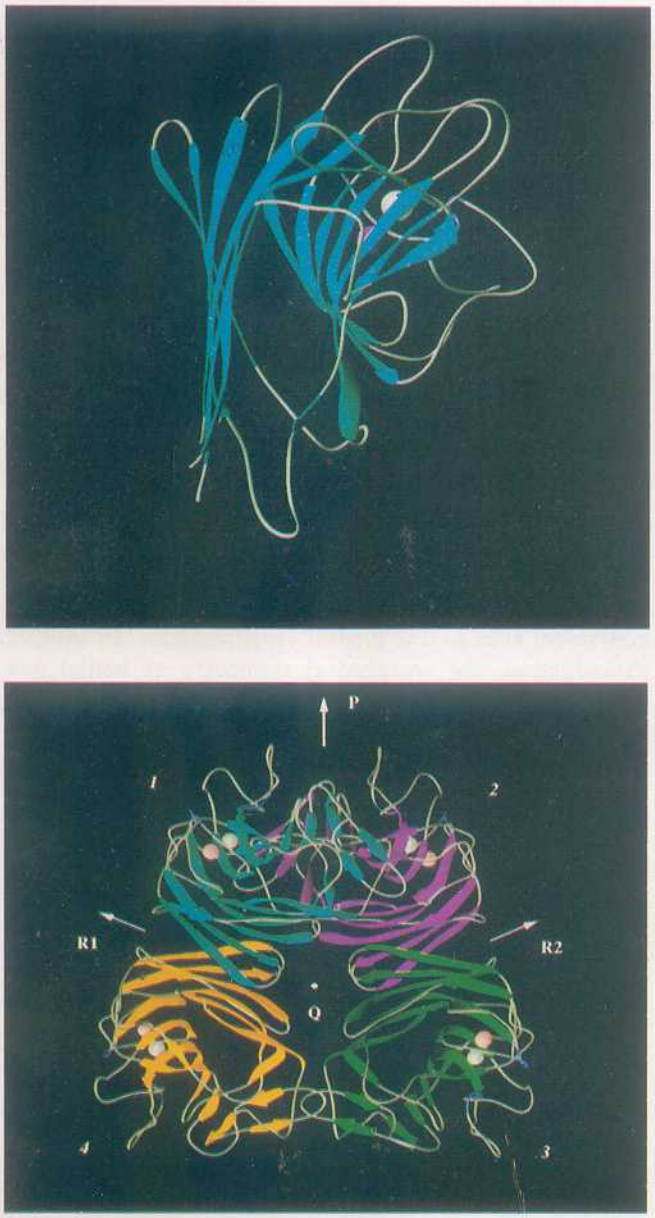


**Figure 11.** *a*, The polypeptide fold in and *b*, the quaternary structure of peanut lectin[61]. The four subunits are coloured differently. The balls represent metal ions.

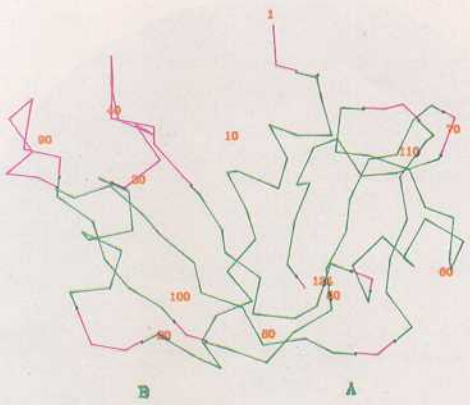


**Figure 12.** Mobility in ribonuclease A[71]. The flexible regions are shown in red in the α-carbon trace. The two domains (A and B) can also move slightly about the hinge connecting them.

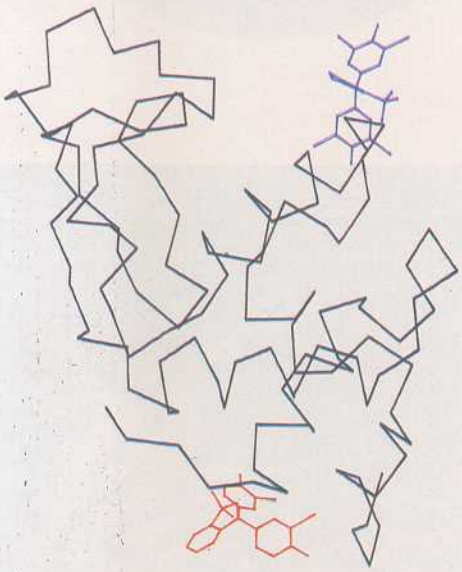

**Figure 13.** The binding sites of bromophenol red (red) and bromophenol blue (blue) in lysozyme[74].

decade to solve in an effort which almost parallelled the development of macromolecular crystallography in India, provides new information on and insights into the polypeptide fold and the carbohydrate-binding properties of legume lectins. However, the most interesting aspects of the peanut lectin molecule, which is of considerable general interest, is its quaternary structure. A well-established principle of subunit association in multimeric proteins is that they should have 'closed' structures with appropriate point group symmetries. Peanut lectin violates this principle and demonstrates that 'open' structures also need to be taken into account when dealing with multimeric proteins. Furthermore, the structure demonstrates that, contrary to earlier belief, the variability in quaternary association in legume lectins is not necessarily caused by interactions involving covalently bound sugar. Indeed, these lectins constitute a family of proteins in which small alterations in essentially the same tertiary structure leads to large differences in quaternary association. This conclusion has been further strengthened by the recent structure determination of the basic winged bean lectin (ref. 62 and unpublished results). The third lectin analysed at Bangalore is jacalin from the seeds of the jackfruit[63,64]. Jacalin, although specific to T-antigen like peanut lectin, has a hitherto unobserved lectin fold and a novel carbohydrate binding pocket (unpublished results). The structure solution of this protein, which has just been completed, is expected to have a major impact on the structural biology of lectins.

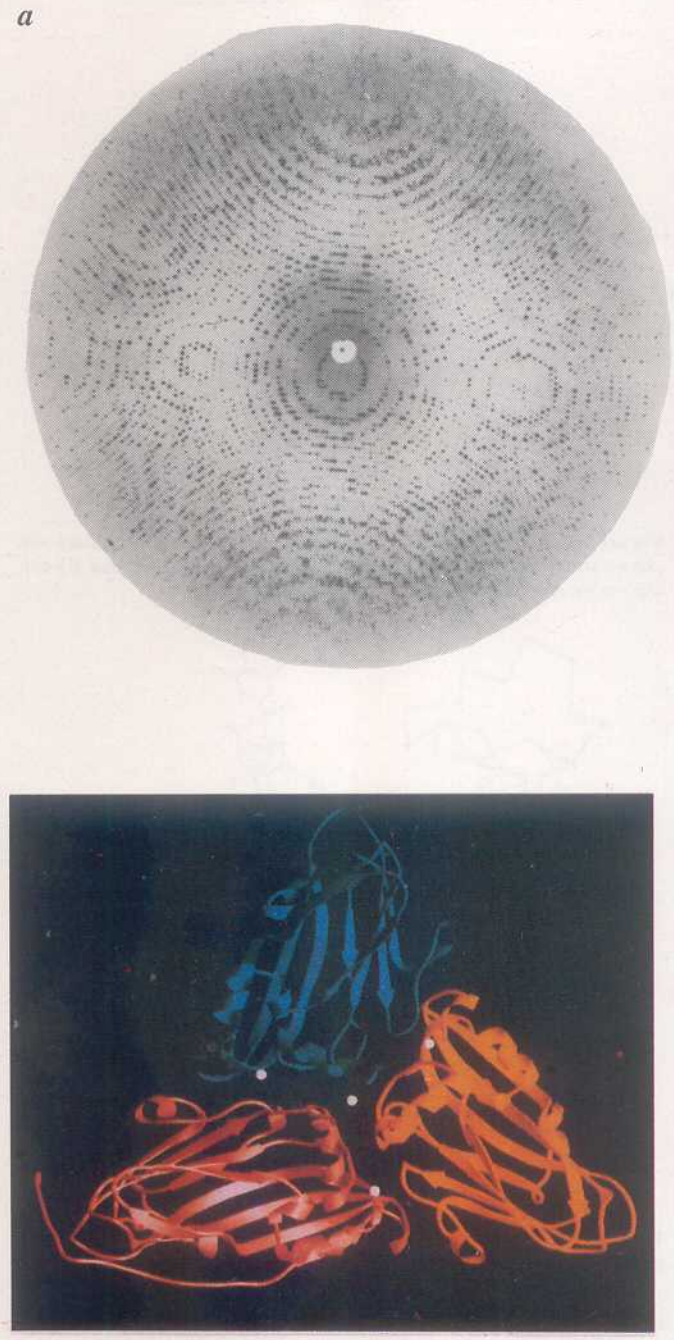

**Figure 14.** *a*, A 1/2° oscillation photograph from the crystals of Sesbania Mosaic Virus, kindly made available by M. R. N. Murthy. *b*, The icosahedral asymmetric unit of the 180-subunit Sesbania Mosaic Virus[76]. The three subunits are coloured differently. The white dots represent calcium ions.

Another long-range programme is concerned with the study of the hydration and mobility of proteins using a novel approach involving water-mediated transformations developed by us[65–67]. This study has led to the elucidation of the nature of the mobility of the well-known enzymes lysozyme and ribonuclease A and the identification of the relatively invariant water molecules in their hydration shell (Figure 12) (refs. 67–71). The results of the study also indicate a strong relationship among dehydration, mobility and enzyme action[71,72]. In yet another programme, two additional binding sites in lysozyme, just outside the hexasaccharide cleft, have been characterized (Figure 13) (refs. 73, 74). They could be important in positioning the enzyme molecule on the bacterial cell wall perhaps through interactions with the peptide component of the peptidoglycan.

Viruses are the largest molecular assemblies that have been successfully analysed using X-ray crystallography. It is indeed gladdening that one of the virus structures solved todate has emanated from India. This structure, that of Sesbania Mosaic Virus which infects *Sesbania grandiflora* plants in Andhra Pradesh, has been solved by M. R. N. Murthy and his colleagues in collaboration with H. S. Savithri and K. Nayudu (Figure 14) (refs 75, 76). In addition to the wealth of information the virus structure provides, the structural work has led to the first experimental demonstration, in relation to calcium binding, of the differences in the chemistry of quasi-equivalent sites in icosahedral virus particles. In another investigation, the icosahedral symmetry of belladonna mottle virus, recently rechristened as physalis mottle virus, has been established[77,78]. It has been demonstrated that this virus is structurally similar to southern bean mosaic virus and cowpea mosaic virus.

The new projects recently initiated by Murthy and his colleagues include one on proteinacious inhibitors of proteolytic enzymes[79]. Murthy and Suguna have also been involved in developments in methodology, particularly in relation to data processing and molecular replacement[78,80,81]. Arising out of this X-ray work, Murthy has also carried out interesting work on structural comparisons and the effect of sequence on structure[82,83].

Yet another project, being pursued by R. Varadarajan and his colleagues, combines protein crystallography, physico-chemical and synthetic studies and mutation analysis to study protein folding in relation to ribonuclease A[84].

Protein crystallographic studies have been initiated recently in the Department of Physics which has had a distinguished tradition in the crystallographic studies, carried out by M. A. Viswamitra and his associates, on nucleic acid components, oligonucleotides and drug–DNA interactions[85–89]. Indeed, the pioneering contributions of Viswamitra in elucidating the basic geometry of nucleic acid constituents and in establishing the sequence-dependence of DNA structure are very well known. The proteins being studied by Viswamitra, S. Ramakumar and others include xylanases and a cellulase[90,91].

The fibre diffraction work on DNA, carried out by V. Sasisekharan in the mid-eighties at MBU[92–94], perhaps constitute the only systematic study of the kind on biopolymers done in recent years in India. The objectives of these studies were to test and elaborate the theoretical contributions that emanated from his laboratory,

particularly in the late seventies and the early eighties, on the alternative structures of DNA.

## Concluding remarks

The most spectacular applications of X-ray crystallography in recent decades have indeed been in the field of biological macromolecules. Macromolecular crystallography has demonstrated how the functions of complex biomolecules and their assemblies are intimately related to their structure. In the process, it has become an essential component of and a decisive influence on modern biology and its myriad applications. Structure determination has now become an indispensable step in any serious biological investigation at the molecular level. The recent emergence of nuclear magnetic resonance (NMR) as an additional tool for macromolecular structure determination has added a new dimension to structural biology. Structural biology in the coming years promises to be as exciting as, if not more exciting than it is now.

It is a matter of some satisfaction that India has a reasonable presence in the field, despite the comparatively late start. The Indian effort in the field is now truly internationally competitive, although we still have to go a long way before we can compare ourselves with the very best in the world. Yet another positive feature is the increased interactions during the last few years between macromolecular crystallographers and biochemists in the country. The state of expertise and facilities have also reached a stage where serious efforts could be initiated on drug and molecular design.


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