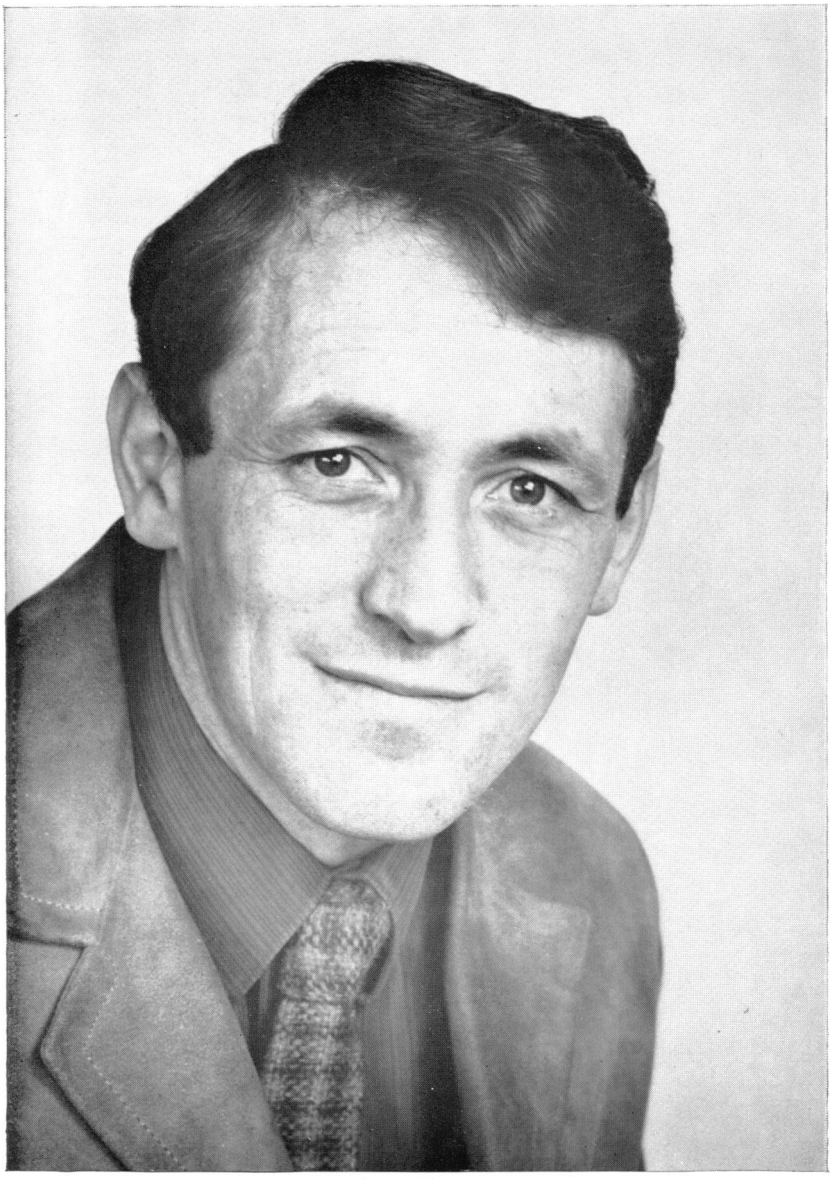

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Biochem. J. (1969) **112**, 261
Printed in Great Britain

The Study of α-Chymotrypsin by X-Ray Diffraction

THE THIRD CIBA MEDAL LECTURE

By D. M. BLOW*

Delivered at a Meeting of The Biochemical Society on 12 September 1968 at University College, Cork, Irish Republic

The Biochemical Society's award of the CIBA Medal for 1967 might be taken to imply two false postulates. The first of these would be that the work for which the medal was awarded was done by me alone. A full list of the graduate research workers who participated in the work appears in Table 1, but I must single out for special mention the collaboration with Dr Brian Matthews (now at the National Institutes of Health, Bethesda, Md., U.S.A.), and Dr Paul Sigler (now at the University of Chicago) during the years 1964–67. The latter part of the investigation that I am going to describe was very much a fruitful collaboration between the three of us, in which we all played parts that were essential to the whole. It was a very great encouragement and pleasure to all of us to hear of the CIBA award.

The second false postulate would be that the work was done in 1967. Professor Manfred Eigen has pointed out that the known time-constants for chemical reactions rarely exceed the time allowed for preparing oneself for the Ph.D. degree. There are certainly examples where a single experimental investigation has lasted much longer than this. The labours of my mentor, Dr Max Perutz, in his investigation of the structure of horse haemoglobin by X-ray diffraction, begun somewhere about 1937 and completed (in the sense that the atomic positions of virtually all the atoms became known) in 1968, form a monument to scientific vision and single-mindedness. I think the particular successes in this field that have been achieved in the United Kingdom are largely due to the willingness of the Medical Research Council to support long-drawn-out investigations of this kind.

This is particularly important because it is a feature of these crystallographic investigations that no useful information is obtained until almost the end of the work. Over the last few years a number of accounts of the basic methods and principles of X-ray diffraction have been aimed at biochemists, but what is much harder to convey is how the time in such an investigation is spent and what people have actually been doing. I think you might like me to spend part of this lecture in rendering an account of that time by mentioning a few of the milestones of the study to indicate which were the actual problems that loomed largest in our minds.

The requirements at present for a successful diffraction study of a protein molecule are, first and foremost, good crystals; and, secondly, a number of isomorphous replacements in which single X-ray-scattering centres—atoms of high atomic number—are substituted into the crystals at unique sites with a minimal disturbance of the remainder of the structure. Two reasons for selecting α-chymotrypsin for study by X-ray diffraction were that it was known to be readily crystallizable (Bernal, Fankuchen & Perutz, 1938) and that there existed a unique site, the serine residue of the active centre, at which substitutions could readily be made (e.g. by di-isopropyl phosphorofluoridate) (Jansen, Nutting & Balls, 1949). Both of these reasons turned out to be misleading.

In 1960 Mr R. J. Pollitt, who was then at the Chemical Laboratory of the University of Cambridge, prepared for us a whole range of analogues of di-isopropyl phosphorofluoridate substituted with a variety of mercury- and iodine-containing groups. These compounds seemed to me at first frighteningly poisonous, but I gradually realized that many of them were relatively insoluble. What neither of us appreciated at that time was that the phosphoryl-ester bond that is made in the combination of di-isopropyl phosphorofluoridate with

Table 1. *Scientific collaborators on the structure of α-chymotrypsin*, 1960–67

D. M. Blow	(1960–)
M. G. Rossmann	(1960–64)
Miss Ann Jury	(1960–61)
R. J. Pollitt†	(1960–61)
Miss B. A. Jeffery	(1961–64)
B. W. Matthews	(1963–66)
P. B. Sigler	(1964–67)
R. Henderson	(1966–)

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the enzyme is sufficiently rapidly hydrolysed at pH 4 (at which pH the crystals are prepared) that it did not survive the length of time required before X-ray-diffraction photographs could be taken.

As progress with this approach was not encouraging, I was trying at the same time to find inorganic ions that would associate with specific sites in the molecule in the same way as had been done with myoglobin. In the middle of 1961, after numerous failures, the first hopeful substituent appeared in the form of the chloroplatinite ion, $PtCl_4^{2-}$. We obtained a series of photographs that allowed us to make a Patterson projection of the difference of electron density with and without the chloroplatinite ion, which suggested that the ion was bound at very localized sites (Fig. 1*a*). No definite interpretation could be made from projections, however.

We had been aware of a difficulty with the crystals that we were working with, which was that they were twinned: that is to say, in one crystal there was a mixture of two types of crystal orientation that could not be separated. Twinning causes X-ray-diffraction photographs to show two patterns superimposed on each other. It was hard to know whether a crystal was twinned simply by examining it under the microscope. As soon as Rossmann and I tried to extend our study into three dimensions this problem became acute. What it meant in practice was that we had to search through large numbers of crystals, taking X-ray-diffraction photographs of one after another, until we found one sufficiently free of twinning to give satisfactory results. Since the planned three-dimensional study at a resolution of 6 Å would need about 30 crystals, this was a large undertaking and took a great deal of time. We also began to run into the problems of data handling for the first time. At that time we were using a computer to which we had access only at night. As what then appeared to be very large amounts of data began to be obtained for these crystals, it became necessary to spend one night a week working with the computer, an unreliable and temperamental 'beast'. Five days and one night of work a week soon began to make me resemble the computer. From this time on, a great deal of effort was devoted to computing problems, but after a year or so we avoided night work.

In 1963 the three-dimensional difference map of the chloroplatinite derivative was obtained (Fig. 1*b*). It showed that the chloroplatinite group was indeed bound to a small number of sites, and I at first interpreted this map as being due to three binding sites. Michael Rossmann showed me that the interpretation could be improved by allocating a fourth site; some years later Brian Matthews found a fifth; now we know that there are six in all.

Meanwhile Rossmann and I had been working on other approaches to protein crystallography that are not really relevant to this lecture, except that they enabled us to draw some deductions about the arrangement of molecules in the chymotrypsin structure that were quite independent of the results of the isomorphous-replacement method. In crystalline chymotrypsin, the repeating unit that is used to build up the crystal structure by the operation of the crystallographic symmetry contains two molecules. Another way of expressing this is to say that the crystals contain two types of molecules, distinguished by being in different environments within the crystal. The method we were studying enabled us to find the relative orientations and positions of the two molecules (Fig. 2). We had our first real success when we found this interpretation confirmed by low-resolution results for the structure of chymotrypsin, obtained from the chloroplatinite derivative and the very closely related iodoplatinite derivative (Blow, Rossmann & Jeffery, 1964).

In 1960, when we started this investigation and the results at high resolution on myoglobin (Kendrew *et al.* 1960) and at low resolution on haemoglobin (Perutz *et al.* 1960) were fresh in all our minds, many of us had the optimistic feeling that all proteins would turn out to be constructed of α-helix, and that all that was needed to obtain a general picture of a protein structure was a three-dimensional map at a resolution of about 6 Å, which would show up the α-helical backbone and the way that it was curled up to make the protein molecule. Our three-dimensional electron-density map, obtained as I have described, was thus very disappointing to us. It showed no sign of α-helix and, apart from the fact that it did appear to confirm the molecular arrangement that we had assumed, had no interpretable features of any kind. We now know that it was very much like a low-resolution map of any other non-helical protein.

At about this time two important discoveries were made that had far-reaching consequences for us. The first, in our own laboratory, was that if small amounts of dioxan were added to the crystallizing mixture the twinning, which had taken up so much of our time and energy over the previous couple of years, was eliminated. We found this by the lucky chance of using dioxan as a solvent for various heavy-metal compounds. The second was the demonstration by Fahrney & Gold (1963) that sulphonyl fluorides acted very much like phosphoryl fluorides as inhibitors of chymotrypsin, but resulted in a very much more stable product. By this time several other groups were working on the structure of chymotrypsins by X-ray diffraction, and in at least three centres the race was on to prepare the obvious heavy-atom analogue of the

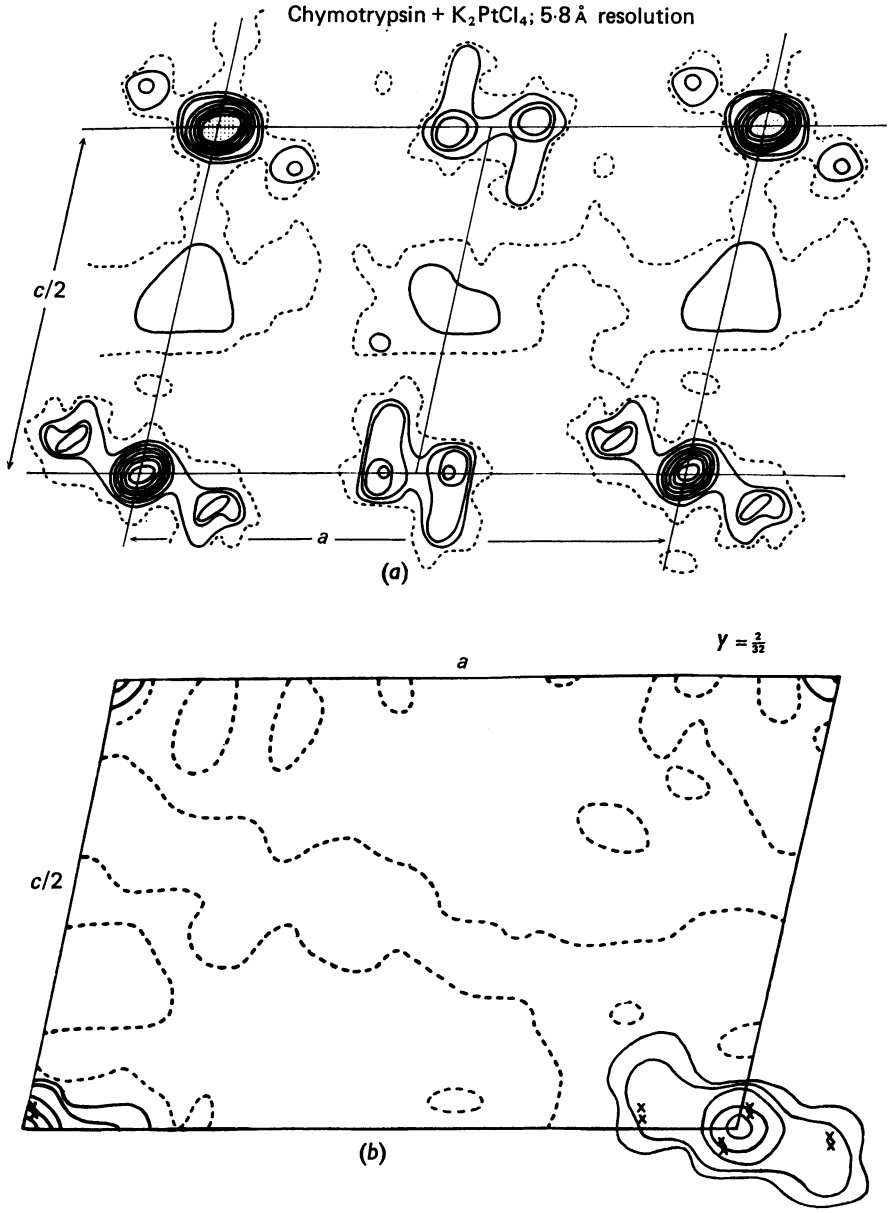


Fig. 1. (*a*) (*h*0*l*) Difference Patterson projection at 5·8 Å resolution for chymotrypsin + K_2PtCl_4. (*b*) Section $y = \frac{2}{32}$ from the 5·8 Å-resolution three-dimensional difference-Patterson function for chymotrypsin + K_2PtCl_4. Crosses mark the positions of heavy-atom vectors (Blow *et al.* 1964).

compound that had been used by Fahrney & Gold (1963), namely *p*-chloromercuribenzenesulphonyl fluoride. Miss B. A. Jeffery in our laboratory discovered a way of preparing this compound, which was also prepared independently in La Jolla in California.

About this time we were joined by Dr Matthews and a little later by Dr Sigler. The situation was in some ways discouraging. It was obvious that we would have to work at high resolution to obtain any interpretable results and yet we were painfully short of isomorphous derivatives. To this situation

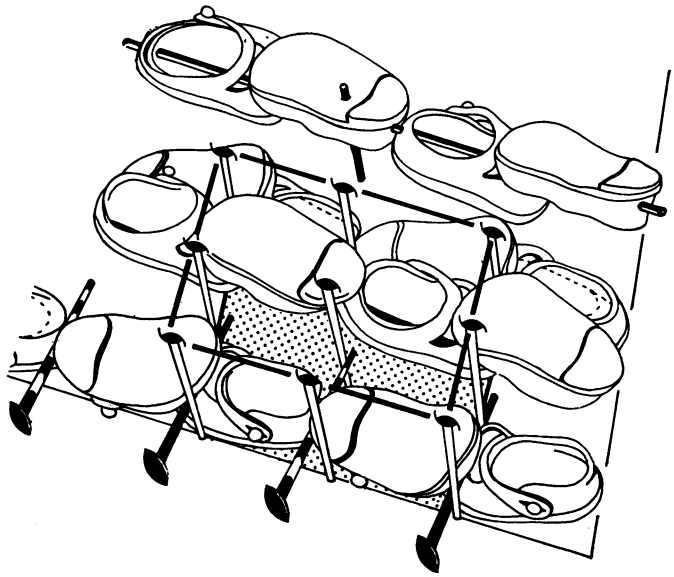


Fig. 2. A model, constructed from asymmetric objects, illustrating the arrangement of molecules found in α-chymotrypsin. The white vertical rods show the positions of crystallographic twofold screw axes. Adjacent pairs of molecules in each horizontal chain are related by a twofold rotation operation about a horizontal axis. These operations are not a part of the crystal symmetry and are known as 'local' or 'non-crystallographic' symmetry axes (Matthews, Sigler, Henderson & Blow, 1967).

Brian Matthews brought experimental and organizational skill that made the practical problems of working at much higher resolution seem feasible, and Paul Sigler brought chemical skill coupled with an enormous enthusiastic optimism that the sulphonyl fluorides, even if the mercury compound was not going to be successful, would provide the answer to our problems of isomorphous replacement.

Over the next year or so we refined our techniques to the point where photographic work to a resolution of 2 Å was perfectly feasible, and we studied a number of the substituents that can be attached to the active centre of chymotrypsin (Sigler, Jeffery, Matthews & Blow, 1966). The mercury compound eluded all attempts to make it bind at a unique site, and continued to give the most miserably poor isomorphism, but the iodine analogue, *p*-iodobenzenesulphonyl fluoride, produced a very beautiful derivative. When it was compared with its own close analogue, the toluene-*p*-sulphonyl derivative, it provided the best isomorphism we had seen. We were able to show that our phase angles at high resolution were sufficiently good to show up the binding sites of the relatively light phosphorus and sulphur atoms involved in the phosphoryl- or sulphonyl-chymotrypsins; and we discovered the fascinating fact that in the dimers that exist in our chymotrypsin crystals the two active centres are only 12 Å apart. It was really at this stage that the attack on the high-resolution three-dimensional structure began.

Here I must interject something to explain in a little more detail what is involved. The decision to collect our diffraction data photographically rather than by diffractometer was forced upon us because no reliable diffractometer was available. However, it turned out to have been a good choice. We were able to collect data much more rapidly than was possible for the haemoglobin group, who had to work with a diffractometer for technical reasons. At the same time, it was a rather large human effort. A typical X-ray-diffraction photograph of chymotrypsin, going out to 2 Å resolution, may include about 2500 diffraction spots. To record the intensity of all the spots on one photograph in the form of a recorder trace takes a girl more than 1 day. A further analysis of the recorder trace to the point where the intensities are punched on to I.B.M. cards and checked takes 3–4 more days. One such film thus represents a full working week. The complete 2 Å data are obtained on a set of about 40 such films. Allowing for failures, errors and annual holidays, it is approximately one girl-year of work to analyse one such set of data. From 1964 to early 1967, we had one, two, and occasionally three assistants working flat out on this task.

In 1965 we had completed this process for the native enzyme and for the chloroplatinite deriva-

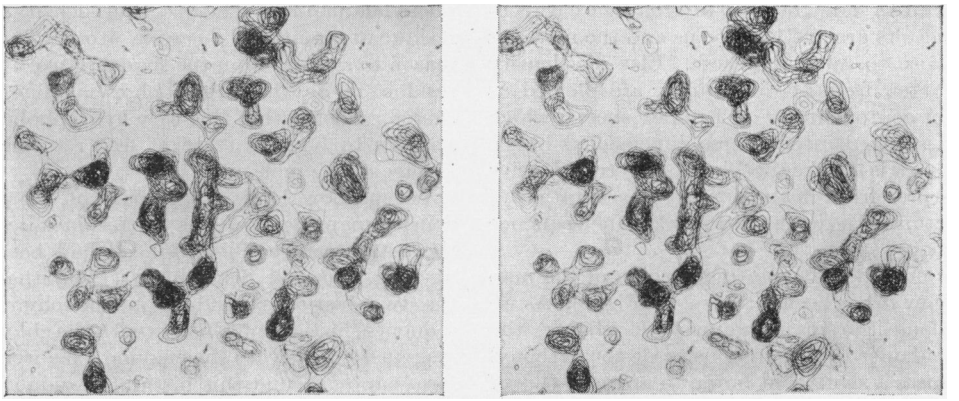


Fig. 3. Stereo pair of diagrams showing the electron-density map of toluene-*p*-sulphonyl-α-chymotrypsin, including sections $x=\frac{19}{64}$ to $\frac{17}{64}$.

tive. We had not expected the map obtained from a single isomorphous derivative to be interpretable, but it was a first test of our complete system. About a year later we were able to add to this a further isomorphous replacement, which was achieved by comparing the *p*-iodobenzenesulphonyl derivative and the closely isomorphous toluene-*p*-sulphonyl derivative. Two whole sets of measurements had to be made to make use of the extremely good isomorphism between these two. Moreover, the difference between an iodine atom and a methyl group is not so large as one would like. This doubly phased map was a great disappointment to me. Remember that we have two independent molecules in the repeating crystallographic unit, giving us two views of the structure. It was my hope that these two views, coupled with the phasing from two isomorphous derivatives, would provide us with enough information to produce an interpretable map, and I was very disappointed to find that this was not so. Meanwhile Sigler had suggested some experiments showing that the ammonium ion, which was present in all our crystallizing solutions, was competing with the protein for many of the heavy-metal derivatives that we had been trying to use as isomorphous derivatives. It proved quite easily possible to replace the ammonium ion by potassium ion in the supernatant of the crystals. Very much better binding of various metal ions was observed, and in this way we obtained a further isomorphous derivative, phenylmercury acetate (Sigler & Blow, 1965). So late in 1966 we knuckled down again to the task of the collection of another set of data and early in 1967 produced a map, phased on three derivatives, which was interpretable (Matthews *et al.* 1967).

I suppose that my story should end there, because this is the point which the published investigation had reached at the end of 1967. I must add that since that time we have tried to improve the quality of that map, first by taking data from a further isomorphous derivative. Although you might think that we were by now experienced hands at this game, this was found to be an expensive waste of time, because the new derivative turned out to be quite useless. However, we also found it possible to carry out very significant improvements in the interpretation of the isomorphous derivatives that were already available to us, and by further careful refinement of the parameters of the isomorphous replacements we have calculated a much improved map during the last few months, which has shown considerably more detail than was available to us in 1967.

There are in α-chymotrypsin three separate polypeptide chains referred to as the A, B and C chains, which are the result of activation and autolysis by trypsin and chymotrypsin. The amino acid sequence as determined by Hartley (1964) and confirmed by Keil, Šorm and their collaborators (Meloun *et al.* 1966) shows that the three chains are held together by two disulphide bridges. Three further disulphide bridges form loops in the B and C chains, which we refer to as the histidine loop, the serine loop and the methionine loop. The histidine loop is so called because the only two histidine residues in the molecule occur very close to each end of the disulphide bridge that forms this loop. There is the sequence -His-CyS- on one side and -Phe-His-Phe-CyS- on the other. The serine loop is so called because it includes the active residue serine-195, and the methionine loop includes one of the two methionine residues in the molecule. The main chain conformation is probably most clearly illustrated by a stereo illustration of a bent wire model, showing the conformation of the different chains in colour (Plate 2*a*). To go further in describing the progress of our interpretation of the

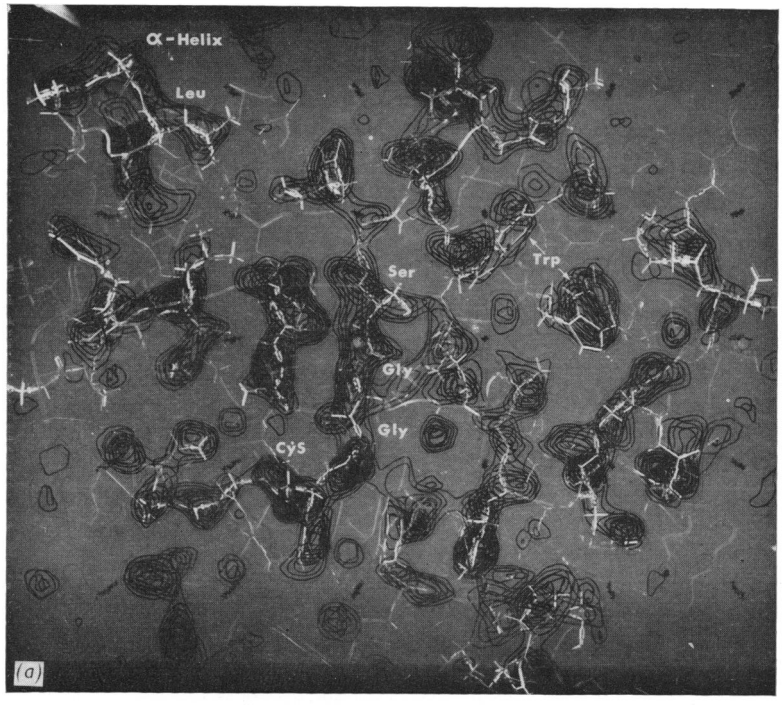
map, I want to show the form in which the electron-density results are available to us and the methods that we use to interpret these. Electron-density values, taken from the computer, are plotted as a series of contour maps on Perspex sheets, which are then stacked into a three-dimensional block. Our task now is to take this block of electron density and interpret from it an atomic representation of the structure. To do this we rely on the sequence information.

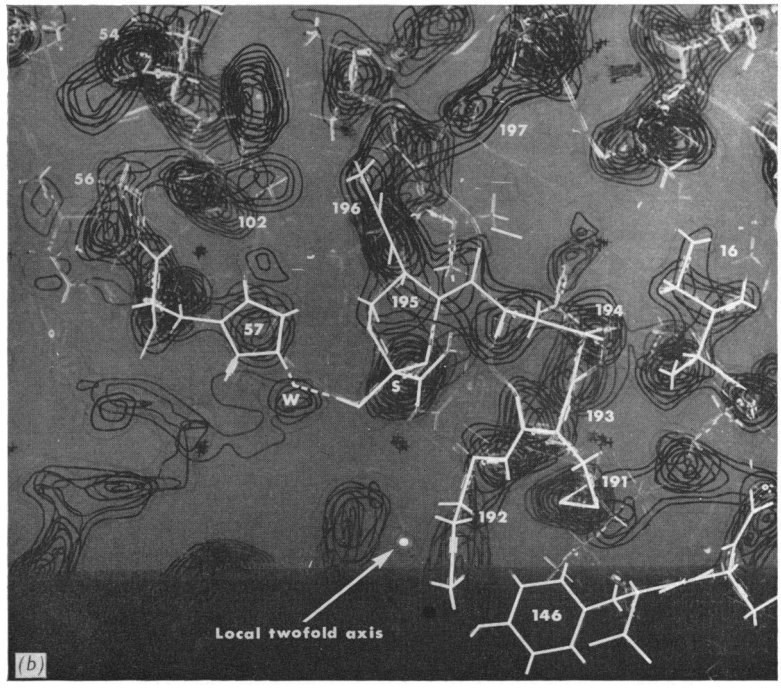
Let us now look at one small region of the map and see how it has been interpreted (Fig. 3). As we follow along the main polypeptide chains, the slightly enhanced electron density at the carbonyl group causes a significant bump to appear. One of the crucial differences between this map and the last is that throughout almost the whole of the polypeptide chain these carbonyl bumps can be seen, and they enable the plane of each peptide group to be known with a fair degree of certainty. In addition to this, the amount of detail shown in the side chain allows a reasonably detailed interpretation of the orientation of each side chain to be made, as indicated in Plate 1(*a*). A section of main chain shows clearly, in which two of the residues involved are glycine, so that the carbonyl group is the strongest electron density involved. The next residue along this chain is serine: its side chain can be seen. There are two tryptophan side chains, which show their general shape very clearly, and a slight dimpling of the six-membered ring can be observed. A very intense pair of peaks, just resolved, is seen in a disulphide bridge. And finally these sections run through a piece of α-helix at the *C*-terminus of the molecule, and the helical arrangement of density can be seen also. Nearly all the shapes of electron density can be reproduced by the atomic model. A leucine residue in the α-helix gives a clear example of a branched side chain. At this stage we have not yet completed the adjustment of the atomic arrangement to fit into the electron density, and slight improvements can still be made. In the map that we made in 1967, there were three regions where we could not be entirely sure of the interpretation and a fourth where we had a considerable amount of difficulty. These have all now been cleared up, with the exception of the conformation of one or two isolated residues. An interesting case appears at one end of the histidine loop, where there is the sequence -Phe-His-Phe-. In 1967 we were able to pick out the second phenylalanine residue and the histidine residue clearly enough, but at this point (as we worked backwards along the chain) our interpretation ceased because we could not identify the first phenylalanine residue. Working in the other direction of the chain we also ran into difficulties. The interpretation is now quite clear except at the first phenylalanine residue, phenylalanine-39. The electron density for this residue stops at the β-carbon atom. There is no reason to suspect from the sequence work that this residue is anything but phenylalanine, and it therefore means that this very hydrophobic residue appears to be in a state of free rotation on the surface of the molecule.

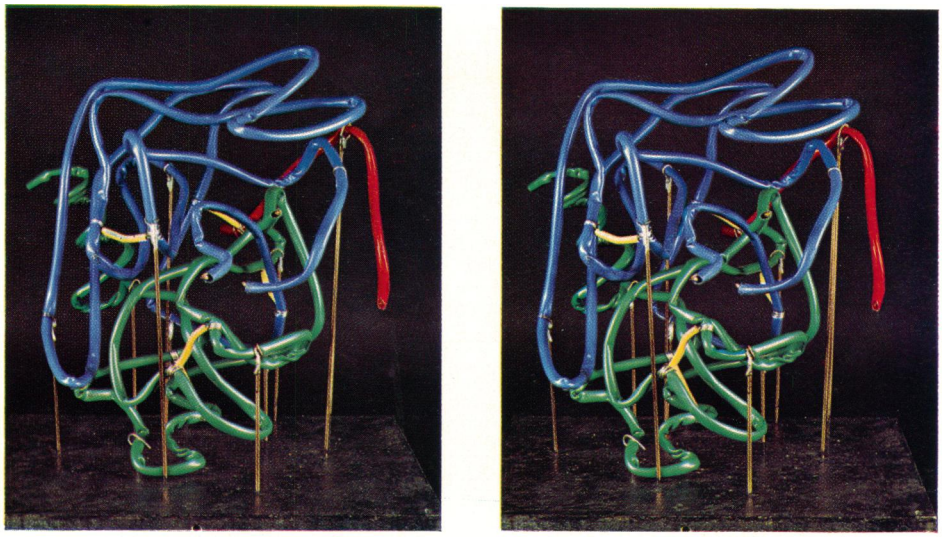
Let us now look at the part of the electron-density map that includes the residues involved in the active centre (Plate 1*b*). It had been shown by Ong, Shaw & Schoellman (1965) that one of the two histidine residues in the chymotrypsin sequence, histidine-57, reacts with the chloroketone reagent to inactivate the enzyme. We were, therefore, happy to find this histidine residue adjacent to the active serine residue, serine-195. The position of the sulphonyl group had already been determined in our studies of the inhibition of the enzyme. The sulphur peak and the peak corresponding to the histidine ring were sufficiently close to make us believe there was a hydrogen bond between residues histidine-57 and serine-195. As one moves along the polypeptide chain from serine-195, the next residue is aspartic acid-194. We were surprised to discover that the side chain of this residue was perfectly clear and pointing more or less into the middle of the molecule. Adjacent to it was another residue which in our 1967 map was one of our clearest isoleucine residues, which turned out to be isoleucine-16. This carries the α-amino group of the B chain, the α-amino group that is liberated on activation of the enzyme by trypsin. We immediately felt sure that we had found the mechanism by which the active centre was held in its active configuration. The existence of an ion pair of this kind was quite different from anything that had been seen in protein structures

EXPLANATION OF PLATE 1

(*a*) The electron-density map of Fig. 3, showing, superimposed upon it, an interpretation as a skeletal atomic model. Features referred to in the text are labelled. Hydrogen bonds are indicated by transparent plastic tubing. (*b*) Part of the electron-density map of sections $x=\frac{17}{44}$ to $\frac{24}{44}$, including the active-centre residues, with the interpretation superimposed. This view is seen parallel to a local twofold axis, one of which is near the bottom centre of the picture. The part of the molecule that lies outside the electron-density map, at the bottom right, is related through the local-symmetry axis to electron density at the bottom left of the picture. Some of the side chains that appear are identified by residue number: threonine-54, alanine-56, histidine-57, asparagine or aspartic acid-102, tyrosine-146, cystine-191, methionine-192, glycine-193, aspartic acid-194, toluene-*p*-sulphonylserine-195, glycine-196, glycine-197. Plates 1(*a*) and 1(*b*) were made by the technique of Richards (1968).


α-Helix
Leu
Ser
Trp
Gly
Gly
Cys
(a)


54
197
56
196
102
16
195
194
57
S
W
193
191
192
146
Local twofold axis
(b)



(*a*)


cys
42
cys
58
his
57
asx
102
ile
99
ser
214
ser
195
try
215
cys
191
gly
216

(*b*)

before, and it was very satisfying that it bore an obvious relationship to a functional interaction in the enzyme. The detailed conformation of the region near residue histidine-57 is now significantly different from that described by Sigler, Blow, Matthews & Henderson (1968) (Plate 2*b*). The histidine ring has been turned over so as to make a hydrogen bond from the other histidine nitrogen atom to residue number 102, which in Hartley's (1964) sequence is asparagine. [Since the lecture was given, this residue has been shown to be aspartic acid (Blow, Birktoft & Hartley, 1969; see postscript).]

Another unexpected feature of the structure was the overall spheroidal shape of the molecule. Unlike the other enzymes whose structures were known at this time, chymotrypsin had no cleft, no jaws, and was a very globular-shaped molecule. For years people had been talking about the hydrophobic slit that would bind the typical substrates of chymotrypsin with their tryptophanyl, tyrosyl or phenylalanyl side chains. But no such feature was obvious to us (see postscript). There are only slight alterations to the conformation of the main chain that we picked out in 1967. The most noticeable difference is in the part of the methionine loop that we found difficult to interpret in the last map. We now find almost one turn of α-helix, i.e. three successive hydrogen bonds of the α-helix type are made. Because the carbonyl-group electron density now enables us to identify the direction of almost every peptide bond, we can now state with much greater confidence where there are hydrogen bonds between main-chain atoms. Fig. 4 shows a summary of the hydrogen bonds that we are reasonably sure of. By drawing the main chain in a zig-zag form it is possible to bring together almost all the residues between which main-chain hydrogen bonds are made. In every case adjacent rows of the figure represent polypeptide chains that are running anti-parallel. The pattern of zig-zag lines, which represents the polypeptide structure in a way convenient to make these hydrogen bonds, consists of two sections of a similar arrangement. In each case there is a series of six anti-parallel lines of the sequence that follows the same pattern, and there are further hydrogen bonds of the anti-parallel type between the sixth line and the first line. This arrangement, which was first noticed by my colleague Dr Brian Matthews, can be described by saying that the structure of chymotrypsin contains two cylindrical surfaces of anti-parallel pleated sheet, although the number of such hydrogen bonds that are made is only a very small proportion of the total possible number, and this is a very partial description of the molecule. It might be worth while to investigate whether there is any indication that the chymotrypsin sequence is a result of a gene-doubling around two similar arrangements of this kind.

Fig. 4 also shows the various types of local hydrogen bonding that are observed, and the two α-helical regions are clearly indicated. It is striking that there is only one case of an isolated hydrogen bond that topologically corresponds to an α-helical hydrogen bond. There are a number of hydrogen bonds to the third-nearest neighbour backwards along the chain. This type of hydrogen bonding corresponds to a regular polypeptide helix known as the 3_{10}-helix (Donohue, 1953).

I would like to comment on the situation that now confronts us in relation to a number of enzymes. We have extremely detailed information about the three-dimensional conformation of the structure. We are able to make some fairly obvious deductions from this, relating the enzymic mechanism to residues that are obviously involved. But to interpret the full detail of enzymic mechanism, to discover the significance of each of the amino acid side chains that contributes towards the total enzymic activity, is a very difficult task. It needs close collaboration between the biochemist, the crystallographer and the physical organic chemist. Perhaps in some cases we shall also need a geneticist to supply mutants to do some of the crucial experiments. We are still a long way from the complete understanding of enzyme mechanism. The crystallographer's unique contribution is to demonstrate the complexity of structure that is involved.

Postscript (*November* 1968). We now know definitely that residue 102 is aspartic acid, so that a second buried acid group is close to the active centre. As has been described in detail elsewhere (Blow *et al.* 1969), we think the function of this residue is to supply an electron to a 'charge-relay system'—a system of hydrogen bonds linking it through the polarizable ring of residue histidine-57 to the hydroxyl group of residue serine-195. This provides an explanation for the properties of the

EXPLANATION OF PLATE 2

(*a*) A bent-wire model showing the general conformation of the polypeptide chains in α-chymotrypsin. The A chain is coloured red, the B chain blue and the C chain green. Disulphide linkages are shown in yellow. (*b*) Skeletal atomic model of toluene-*p*-sulphonyl-α-chymotrypsin, showing the active-centre region. The viewpoint of this picture corresponds to the bottom of Plates 1(*a*) and 1(*b*). Plates 1(*a*) and 1(*b*) are looking 'down from the top' of the model; here we look 'in from the side'. The sulphur atom of the toluene-*p*-sulphonyl-serine-195 is shown in dark green, nitrogen in blue and oxygen in red. The cystine bridges are dark green and yellow. Large red and blue shapes are attached to the carboxyl and α-amino groups of the ion pair between aspartic acid-194 and isoleucine-16.

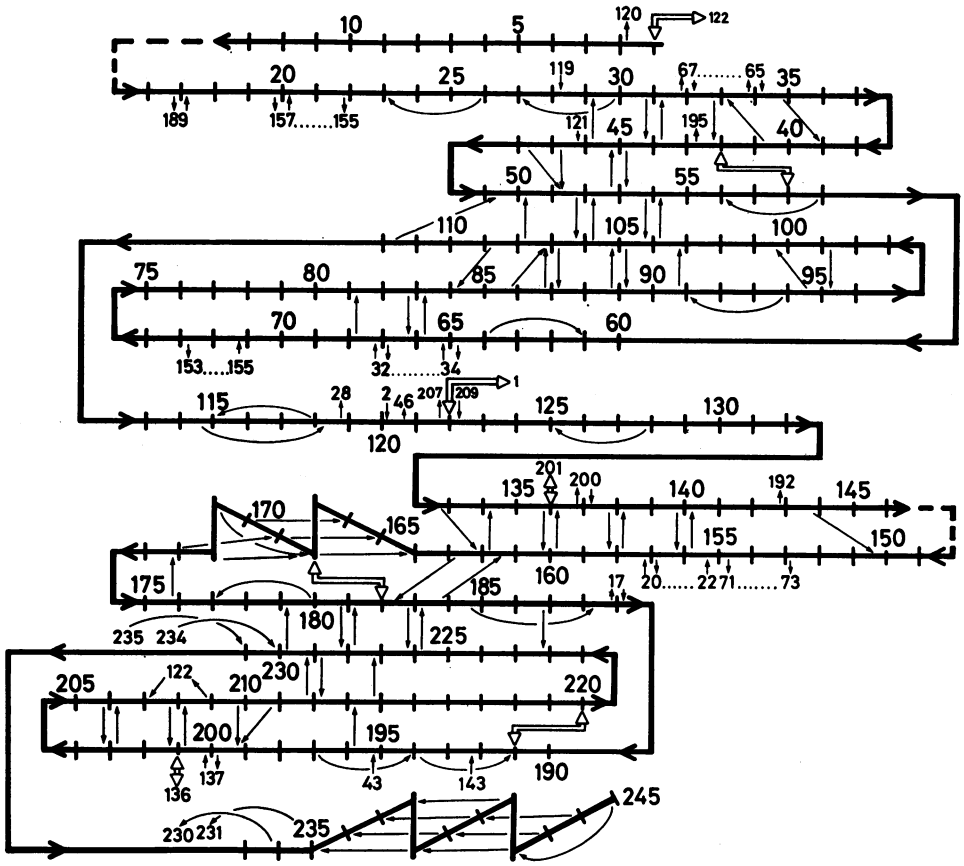


Fig. 4. Hydrogen bonds between the main-chain peptide bonds in α-chymotrypsin. The black arrows (⟶) representing hydrogen bonds are drawn from amido to carbonyl group. The white arrows (⟸⟹) represent disulphide bridges.

serine as a powerful nucleophile, which appears consistent with many other observations about the catalytic properties of the enzyme.

The substrate binding problem also seems to be solved. R. Henderson & T. A. Steitz have shown that, after all, *N*-formyltryptophan will bind to the crystals in essentially the position occupied by the toluenesulphonyl group in Plate 2(*b*).

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