*Acta Cryst.* (1990). B46, 63–69

# Refinement of Triclinic Lysozyme: II. The Method of Stereochemically Restrained Least Squares

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(*Received* 15 *February* 1989; *accepted* 7 *August* 1989)

## Abstract

Refinement of triclinic lysozyme by restrained least squares against the 2 Å resolution X-ray data is described, beginning with the model from cycle 17 of the preceding paper [Hodsdon, Brown, Sieker & Jensen (1990). *Acta Cryst.* B46, 54–62]. After 20 refinement cycles, $R$ stood at 0·172. Nevertheless, serious errors involving both main-chain and side-chain atoms still remained, requiring numerous model rebuilding sessions interleaved with refinement cycles. After 63 cycles $R = 0{\cdot}124$ for the model which includes all protein atoms, 249 water oxygen sites and five nitrate ions. Although the overall $B$ is relatively low, 10·5 Å$^2$, $B$'s for atoms in the region of residues 101–103, toward the termini of some of the longer side chains, and in the region of the C terminus of the main chain exceed 20 Å$^2$, indicating relatively high atomic mobilities, disorder, or remaining errors in the model.

## Introduction

The refinement of triclinic hen egg white (HEW) lysozyme reported in the preceding paper (Hodsdon, Brown, Sieker & Jensen, 1990) was carried through by Fourier and least-squares methods, interleaving model idealization with successive refinement cycles or series of cycles to maintain a stereochemically reasonable model. Except in the earlier cycles, refinement was slow, and when the usual crystallographic $R$ value had been reduced to 0·220 for the idealized model, 1·5 Å data, a systematic error was discovered in the higher-resolution data. The effect of the error on the data to 2 Å resolution was relatively small, however, and a reasonable correction could be applied. In the last five cycles reported in the previous paper, refinement was by block-diagonal least squares against the corrected 2 Å data with idealization of the model following each cycle.

A preferable refinement procedure is to include known molecular parameters such as bond lengths and angles as additional observations in the least-squares alogrithm itself as proposed by Waser (1963). When such a program for protein refinement was announced (Hendrickson, 1976), the refinement by block-diagonal least squares with interleaved idealization was terminated. We report here the refinement of triclinic lysozyme by the method of stereochemically restrained least squares (SRLSQ, Hendrickson & Konnert, 1980), beginning with the model from cycle 17 of the earlier refinement (Hodsdon *et al.*, 1990).

## Data and model

The crystallographic data set for the SRLSQ refinement is the same as that used in the final stages of the earlier refinement, except that initially it included all 7131 reflections with $d$ spacings in the range $\infty$–1·97 Å. During the latter stages of the present refinement, the data set was reduced to 7075 reflections by deleting the 56 reflections with $d \geq 10$ Å. The data were scaled by the same factor, $k = 1{\cdot}455$, as in the final stages of the earlier refinement.

The initial model for the SRLSQ refinement was the one from cycle 17 of the preceding paper, but modified by deleting the 332 water O atoms and replacing the individual atom $B$ values by an overall $B = 8$ Å$^2$. For this model $R$ was 0·314.

The relatively large root-mean-square deviations from ideal parameters listed for this model in Table 1 are not surprising in view of the fact that it was one free refinement cycle beyond the last idealization (see Fig. 2 in the preceding paper).

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0108-7681/90/010063-07$03.00

Table 1. *Refinement details*

Root-mean-square deviations between the model and the ideal parameters for each type of restraint are listed first for the initial model, then for each stage the assigned $\sigma$'s are listed along with the r.m.s. deviations for the models at the end of each stage.

| | Initial model* | Stage 1 | | Stage 2 | | Stage 3 | |
|---|---|---|---|---|---|---|---|
| Type restraint | R.m.s. dev. | Assigned $\sigma$ | R.m.s. dev. | Assigned $\sigma$ | R.m.s. dev. | Assigned $\sigma$ | R.m.s. dev. |
| Covalent bonds | 0·139 Å | 0·05 Å | 0·036 Å | 0·03 Å | 0·022 Å | 0·02 Å | 0·016 Å |
| 1–3 distances | 0·245 | 0·10 | 0·085 | 0·04 | 0·048 | 0·03 | 0·034 |
| 1–4 distances | 0·321 | 0·10 | 0·204 | 0·05 | 0·193 | 0·05 | 0·047 |
| Deviations from planes | 0·075 | 0·05 | 0·055 | 0·02 | 0·032 | 0·02 | 0·018 |
| Chiral volume | 0·746 | 0·40 | 0·246 | 0·15 | 0·163 | 0·15 | 0·140 |
| Single torsion, NB contact | 0·334 | 0·50 | 0·229 | 0·50 | 0·206 | 0·30 | 0·170 |
| Multiple torsion, NB contact | 0·341 | 0·50 | 0·414 | 0·50 | 0·340 | 0·30 | 0·238 |
| Possible H bond | 0·214 | 0·50 | 0·529 | 0·50 | 0·410 | 0·30 | 0·187 |
| Planar torsion angle | 17·8° | - | 14·8° | 5·0° | 6·9° | 3·5° | 4·4° |
| Staggered torsion angle | 23·9° | — | 24·2° | 15·0° | 21·3° | 12·5° | 13·1° |
| Orthogonal torsion angle | 21·8° | — | 18·9° | 15·0° | 17·5° | 20·0° | 19·4° |
| $R(F)$ | 0·314 | | 0·169 | | 0·172 | | 0·124 |

*This model is the one after cycle 17 of the preceeding paper, one cycle after model idealization.

## SRLSQ refinement

*Stage* 1

This stage of the refinement was carried out at Seattle on CDC 6400, CYBER 73 and CYBER 173 computers, and at Bombay on a DEC 1077 computer. The programs used were June and October 1977 versions of *PROTIN* and *PROLSQ* (Hendrickson, personal communication). Since the memory available on the CDC computers was insufficient to refine the model in a single pass, extensive modification of the programs was necessary in order to refine in multiple passes, usually three to five for each full cycle. The program was also modified to deal with partial, but fixed occupancy solvent atoms. Finally, a criterion was incorporated in the computation to treat the relative weights of the observations and restraints on the basis of the sums of the weighted-squared deviations of various kinds. The standard deviations assigned to the restraints at each stage of the refinement are listed in Table 1. The initial weights applied to the X-ray data were taken as $1/\sigma^2$ and scaled by the factor 1/24 so that $\sum w(F_o - F_c)^2$ was approximately equal to the sum of the weighted-squared differences between the model and ideal restraints. As the refinement progressed, the factor was adjusted to maintain approximate equality. Although weights based on counting statistics were applied initially to the X-ray data, unit weights were used in the later stages. The version of the programs used at the time restrained only the bond and angle distances, planes, chiral volumes and nonbonded contacts.

While the initial model for the SRLSQ refinement was unsatisfactory in terms of the stereochemistry, its geometry was largely restored in the first two SRLSQ refinement cycles in which 3005 parameters ($x$, $y$, $z$ for the protein atoms, an overall $B$, and the scale factor) were adjusted against the 7131 reflections in the full 2 Å data set and approximately 3800 restraints. $R$ increased slightly in the first cycle (see Fig. 1), then decreased in the second cycle, but only to 0·309. The 125 sizeable peaks in the solvent region of an $F_o - F_c$ map were assumed to represent water molecules and were added to the model as O atoms with $B$ values of 8, 10, 12 and 15 Å$^2$. The map also provided clear evidence that the $B$'s of many protein atoms were less than 8 Å$^2$. Accordingly, estimated $B$'s in the range 5–10 Å$^2$ were assigned to the individual atoms. $R$ for this model was 0·277, decreasing to 0·244 in the third refinement cycle in which free rotation around the $C_i$—$N_{i+1}$ bonds was allowed by restraining only the four peptide atoms ($C^{\alpha}_i$, $C_i$, $O_i$ and $N_{i+1}$) to be planar, *i.e.*, the $\omega$ angles were no longer restrained. This action was prompted by Ramachandran & Kolaskar's analysis (1973) of the planarity of the peptide linkage which showed that significant deviations from planarity could occur with little increase in energy.

On the basis of a second $F_o - F_c$ map, 108 additional water sites were added to the model and corrections made in the $B$ values of some of the protein atoms, reducing $R$ to 0·229. The next cycle, the fourth, reduced $R$ to 0·218. At that point occupancy parameters were assigned to the individual solvent atoms on the basis of peak heights in difference maps, and refinement continued by cycles of SRLSQ interleaved with $F_o - F_c$ maps to monitor the results. Following cycle eight, restraints on nonbonded contacts were removed (see Fig. 1) to eliminate any possibility of bias arising from the operation of restraints limited to atoms within the molecular segment being refined in a given pass. When $R$ reached 0·170, the model had been subjected to 12 full refinement cycles and the solvent to an additional four cycles (not shown in Fig. 1). At that point the model comprised 1001 protein atoms and 239 solvent O atoms, all with individual $B$ parameters, each solvent atom having, in addition, a fixed occupancy parameter.

A chance calculation of the conformational angles after cycle 12 indicated substantial error in the model although $R$ had been reduced to 0·170. The most disquieting feature was an $\omega$ of 92° between Gly104 and Met105. Accordingly, restraints were reimposed on the nonbonded contacts and on the $\omega$'s by including the $C^{\alpha}_{i+1}$ atoms in the planar peptide linkages. In the following refinement cycle, the thirteenth, $R$ increased from 0·170 to 0·177 and the errant $\omega$ increased to 127°.

The next two refinement cycles were executed on the DEC 1077 computer at TIFR, Bombay. The memory capacity of this machine was sufficient to refine the entire model, protein and solvent, in a single pass. In cycle 14, $R$ increased slightly, to 0·179; removal of $B$ restraints in cycle 15 led to a decrease to 0·169. The errant $\omega$ had increased to 135°.

*Stage* 2

In the second stage of this study, the refinement of HEW lysozyme was continued on the IRIS 80 computer at VECC, Calcutta, using the December 1978 versions of the *PROTIN* and *PROLSQ* programs (Hendrickson, personal communication). Increased weights were assigned to the restraints on distances, planes, chiral volumes and nonbonded contacts, and restraints were imposed on all $\omega$ and side-chain torsion angles (see Table 1). $R$ increased in cycle 16 to 0·184, then changed only slightly in the next two cycles, but after again removing restraints on the $B$'s, decreased to 0·176 in cycle 19 and to 0·172 in cycle 20.

Despite the fairly low $R$ value, many torsion angles in a number of side chains were unsatisfactory, particularly in arginine residues, but also in some aspartic acid, asparagine, glutamic acid and glutamine residues. Additional refinement tests on the DEC 1077 in Bombay did not appreciably improve the model.

At this point a geometric analysis of the model identified ~156 protein and 76 solvent atoms as unsatisfactory. Correcting these on the basis of $F_o - F_c$ and $2F_o - F_c$ maps followed by refinement on the PRIME 450 computer at BARC, Bombay, was slow, and despite an intense effort, progress was minimal. It was now clearly evident that repeated checking and rebuilding on the basis of Fourier maps would be essential if the model were to be further improved by refinement.

The checking of the model and test refinements following cycle 20 are not plotted in Fig. 1 because the final stage of the refinement returned to the model from cycle 20.

*Stage* 3

The availability of an interactive graphics display, a VG 3400 operating under the *FRODO* system of programs (Jones, 1982), and the ND-560 computer

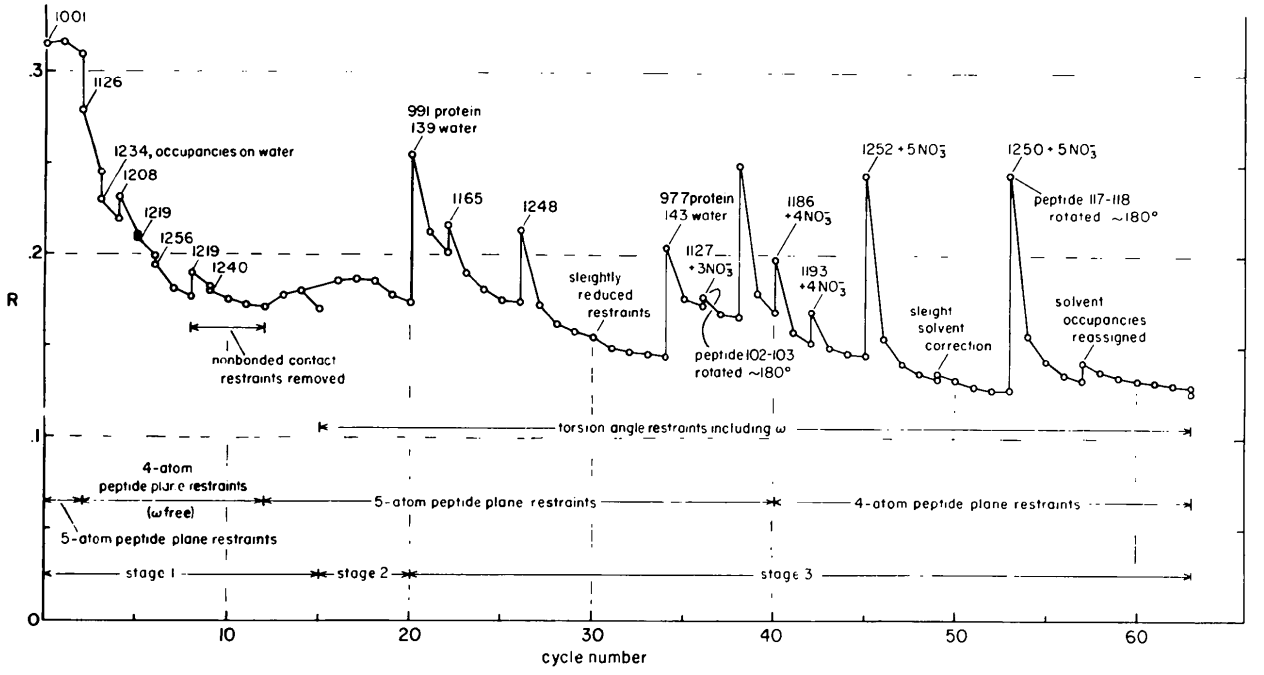


Fig. 1. Plot of $R$ *vs* cycle number. Abrupt changes at a given cycle result from editing the model. Each number appearing above the plot and joined to a point, *e.g.*, 1126 at cycle 2, is the total number of atoms in the model at that point, and it remains the same until a different number appears. Unless otherwise indicated, it is the sum of the 1001 protein atoms and the number of water O atoms in the model.

at BARC, Bombay, greatly facilitated our efforts to further refine the triclinic lysozyme model. The general approach during this stage of the study was to check the geometry of the current model, delete all atoms involved in unsatisfactory geometry, compute $F_o-F_c$ and $2F_o-F_c$ maps based on the truncated model, then add back as many of the missing atoms as possible by fitting to the electron densities on the display, followed by two to four refinement cycles. The pronounced spikes during the latter part of the refinement shown in Fig. 1 (cycles 38, 45, 53) are mainly due to resetting $B$ values for the protein atoms to 8 Å$^2$ and the solvent atoms to 12 Å$^2$. Refinement was continued until it was judged satisfactory at $R = 0{\cdot}128$. Application of a rescale factor and an overall $\Delta B = 2{\cdot}0$ Å$^2$, evaluated outside the refinement program, reduced $R$ to 0·124.*

In stage 3 twelve editing sessions and 43 refinement cycles were executed. Restraints were applied on all parameters, except the solvent–solvent distances, and from cycle 41 onward, the restraints that had been reimposed on $\omega$ in cycle 13 by including $C^{\alpha}_{i+1}$ in the peptide plane were removed, while retaining the torsion-angle restraints imposed on $\omega$ in cycle 16.

Progress in refining the triclinic HEW lysozyme by SRLSQ has been reported periodically (Ramanadham, Hodsdon, Brown, Sieker & Jensen, 1980; Ramanadham, Sieker, Jensen & Birkness, 1981; Ramanadham, Sieker & Jensen, 1985, 1987). Certain aspects of the refined model, especially those pertaining to the antibody recongition epitopes on lysozyme, have been published elsewhere (Ramanadham, Sieker & Jensen, 1989).

## Discussion

### *General comments*

In comparing the earlier differential difference refinement against the 1·5 Å data with the SRLSQ refinement against the 2 Å data, it is evident that the latter was more effective in improving the model. In the first phase of the SRLSQ refinement, $R$ fell rapidly (see Fig. 1), decreasing to less than 0·2 in only six cycles and to 0·17 by cycle 12. The main-chain geometry improved considerably, and although the lack of any restraints on the $\omega$ angles in cycles 3–12 caused some problems, most $\omega$'s adjusted toward expected values. For example, the angle $\omega$ between Phe34 and Glu35 which had been 61° in the initial model adjusted to 172° by cycle 15, and most other $\omega$'s were within ±10° of expected values. Experience suggests that early in refinement weaker restraints can be advantageous, but caution must be exercised to check that no region of the model drifts beyond the convergence range.

It was clearly evident at the end of stage 2 that the model for triclinic lysozyme still suffered errors of such magnitude, even at $R = 0{\cdot}172$, that they could not be corrected by the routine application of SRLSQ. The intensive checking of the model against $F_o-F_c$ and $2F_o-F_c$ maps and test refinements after cycle 20 (not shown in Fig. 1) indicated that progress could be made only by manual intervention to adjust regions of the model that were unsatisfactory. A similar conclusion had been reached early in the differential difference refinement against the 1·5 Å data reported in the previous paper.

In the present refinement, side chains of Arg5, Asn19, Gln41, Asn44, Arg45, Arg61, Arg68, Arg73, Asp101, Asn103, Arg112, Gln121, Arg125, Arg128 and Leu129 were all corrected during the final editing sessions of stage 3. As in the earlier refinement, Arg side chains proved troublesome and along with those of Gln41 and Gln121 were particularly difficult to adjust. At least five to six editing sessions were required before satisfactory results were achieved.

As noted in the preceding paper, in structures such as triclinic lysozyme where most of the solvent is ordered, distinguishing side-chain atoms from solvent can present a serious problem. This is illustrated in Fig. 2(*a*) which shows the side chain of Arg5 and what was presumably adjacent solvent. The electron density of the side chain appears reasonable, but the torsion angles $\chi^4 = -93°$ and $\chi^5 = 143°$ were questionable. After model editing and final refinement, the side chain moved into what had appeared as solvent in Fig. 2(*a*), and the density of the incorrectly oriented part of the side chain virtually vanished, Fig. 2(*b*).

In the earlier refinement (Hodsdon *et al.*, 1990), the electron density in the section of chain from residues 65–73 was at first comparatively weak, and Pro70 could not be recognized in either $F_o-F_c$ or $2F_o-F_c$ maps until near the end of the study. In the SRLSQ refinements on the other hand, this whole section of the main chain from residues 65–73 adjusted into position without difficulty. Presumably the restraints linking the residues in the restrained refinement served to carry Pro70 into position.

The stretch of main chain between residues Val99 and Met105 eluded correction until the latter stages

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 2LZT, R2LZTSF), and are available in machine-readable form from the Protein Data Bank at Brookhaven or one of the affiliated centres at Melbourne or Osaka. The data have also been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 37030 (as microfiche). Free copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England. At the request of the authors, the list of structure factors will remain privileged until 1 December 1990.

of the refinement. Since the electron density was poorly defined in this region, fitting the model proved difficult even though the density had improved somewhat as other parts of the model were corrected. Ultimately a rotation of ~170° around the $C^{\alpha}102$—C102 bond moved the side chain of Asn103 into density which had earlier been treated as solvent. This resulted finally in what seems to be the correct model for this part of the main chain although one must still allow for possible error or disorder in this region.

The added restraints on $\omega$ imposed through the five-atom peptide planes were removed in cycle 41, and only the torsion-angle restraints were retained to control the $\omega$'s. On removing the restraints, an error involving Gly117 and Thr118 was immediately evident which ultimately required a rotation of ~180° about the $C^{\alpha}117$—C117 bond.

*Water model*

The water model was completely redetermined and heavily edited during the refinement. Fig. 3 shows three typical, high-occupancy water molecules. Of the 249 water sites in the final model, 128 have occupancies greater than 0·8, the remaining 121 range downward to 0·4. The occupancies of the present water sites add up to the equivalent of ~194 water molecules, accounting for approximately two thirds of the ~300 water molecules in the unit cell.

A problem in the earlier refinement concerned the group scale factors $k'$ that were found to vary systematically with sin $\theta/\lambda$ (see Fig. 5, Hodsdon *et al.*, 1990). The variation is much less for the results from the present refinement, consistent with the redetermined and much improved solvent model.

*Nitrate ions*

As water sites were added to the solvent model in the refinement, several regions of excess density were observed which were not at acceptable protein–solvent or solvent–solvent distances. In the $F_o - F_c$ map of one such region which had been modeled as a single water site, three peaks (shown in Fig. 4) appeared. The three O atoms of a nitrate group nicely fit the density. Fig. 5 shows the density at this site in the final $2F_o - F_c$ map where the triangular density of the nitrate ion is readily recognized. Five $NO_3^-$ sites are included in the present model and with unit occupancies lead to $B$ values in the range 16–30 Å$^2$.

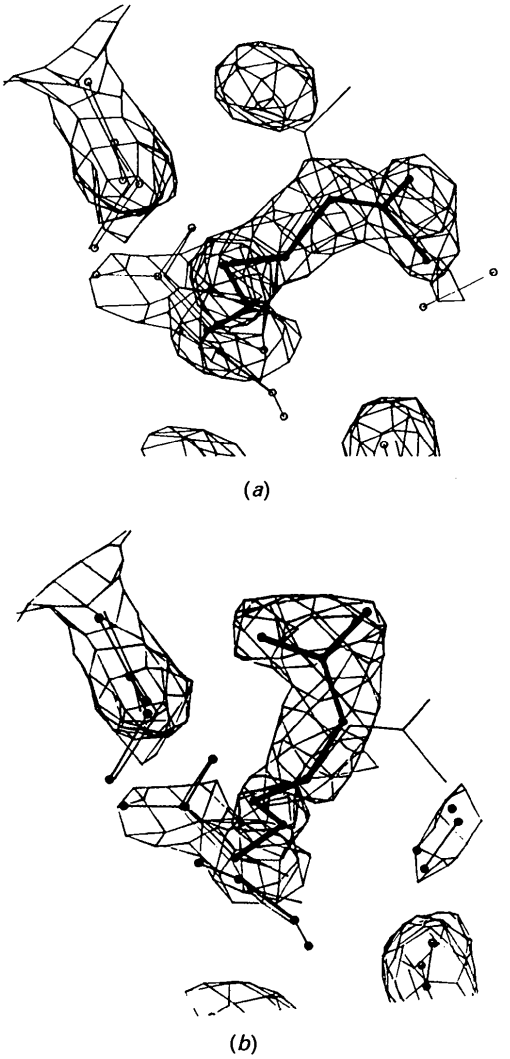


Fig. 2 (*a*) Arg5 residue in a $2F_o - F_c$ map where terminal atoms of the side chain are incorrectly oriented. (*b*) Correct orientation of Arg5 side chain.

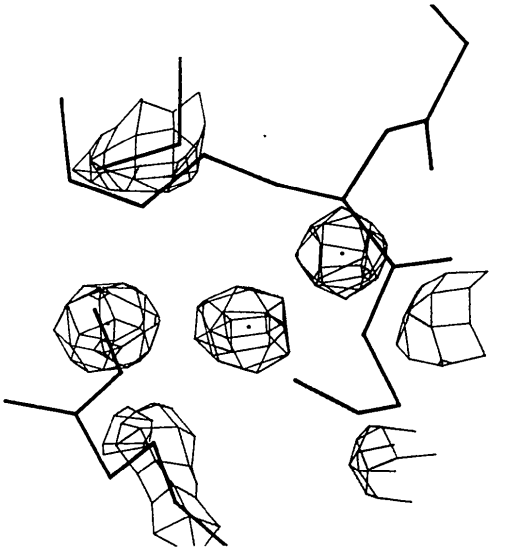

Fig. 3 Three typical highly occupied water molecules in the $2F_o - F_c$ map based on final phases.

*B values*

The distribution of main-chain $B$'s is shown in Fig. 6. The average $B$ for all protein atoms is 10·5 Å$^2$, and for main-chain and side-chain atoms separately the values are 9·1 and 11·9 Å$^2$ respectively. These values are in good agreement with the $B$'s obtained in a normal-mode dynamic calculation by Levitt, Sander & Stern (1985).

The main-chain $B$'s of the first 98 residues are, in general, considerably less than those of the last 31. The distribution is consistent with the secondary structural features of the lysozyme molecule (compare Fig. 6 with the stereoview of the molecule in Fig. 7). Large thermal motion or possible disorder is associated with residues in the region of 100–103, 117 and 128–129. Side chains of most Arg residues and some others, particularly Asp, Asn, Glu and Gln residues, are also associated with large $B$'s and may suffer disorder or even error.

The side chain of Trp62 is anomalous in the sense that the $B$'s increase abruptly beyond $C^\beta$ and remain high for all nine atoms, ranging from 23–29 Å$^2$, in contrast to a range of 4–7 Å$^2$ for the corresponding atoms in the adjacent Trp63. The remaining three Trp residues in the molecule are in the C-terminal region of the chain where the $B$'s are, in general, relatively high (see Fig. 6). Despite this, $B$'s for the ring atoms in Trp108, Trp111 and Trp123 are relatively low, ranging from 8–9, 6–8 and 7–11 Å$^2$ respectively.

The large $B$'s for the Trp62 side chain are in accord with the low, irregular electron density in a $2F_o - F_c$ map. The density is not readily accounted for on the basis of disorder. It appears more likely that this side chain has been damaged, either directly or indirectly by the radiation or by some environmental factor during the extraction and purification of the material. In fact, it is known that ozone selectively attacks the side chain of Trp62 in HEW lysozyme, rupturing the $C^\gamma$—$C^{\delta 1}$ bond (Sakyama & Natsuki, 1976).

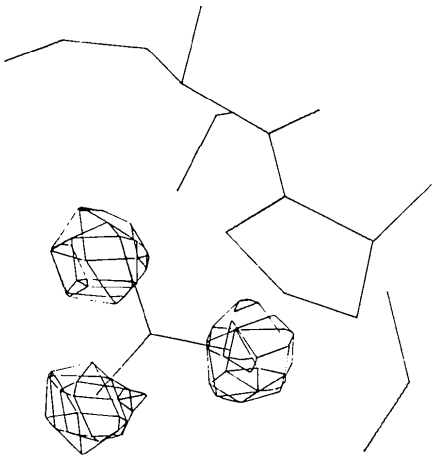

Fig. 4. $F_o - F_c$ map showing three O atoms of a nitrate ion in the vicinity of Pro70. The central atom of the nitrate ion was included in the water model so that it does not appear in the $F_o - F_c$ map.

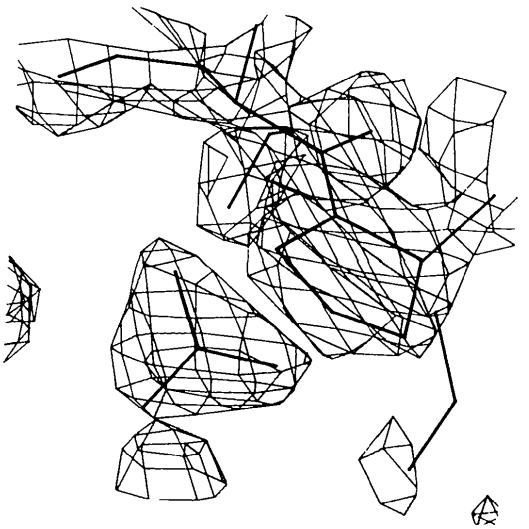

Fig. 5. The nitrate ion near Pro70 as it appeared in the final $2F_o - F_c$ map.

## Concluding remarks

The present refinement of HEW lysozyme shows that errors as serious as misoriented peptide groups remained in the model at $R = 0{\cdot}172$. The most pervasive errors, however, involved the side chains of many residues (*e.g.*, see Fig. 2). Rebuilding the model on the basis of $F_o - F_c$ and $2F_o - F_c$ maps with interleaved cycles of SRLSQ refinement, followed by

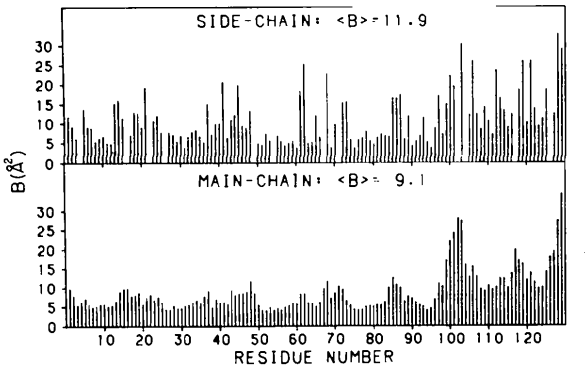


Fig. 6. $\langle B \rangle$ for the four main-chain atoms of each residue as a function of residue number (lower plot). Similarly, $\langle B \rangle$ for the side-chain atoms as a function of the residue number is shown in the upper plot.

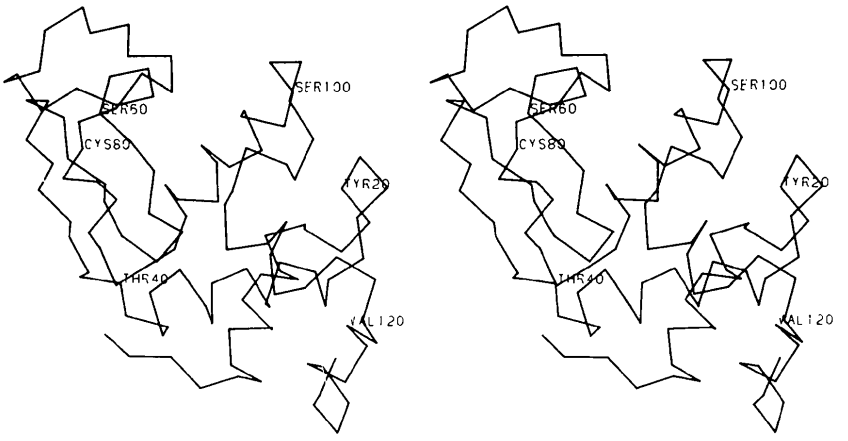


Fig. 7. Stereoview of the $C^{\alpha}$ chain of the lysozyme molecule based on the coordinates from the final refinement cycle of triclinic lysozyme.

applying an overall $B = 2{\cdot}0$ Å$^2$ and rescaling, reduced $R$ to 0·124 for the 2 Å data. Clearly, the model has been much improved, and it is probably approaching the limit that can be expected at 2 Å resolution.

The crystallographic index $R$ is customarily cited as a measure of the acceptability of models based on X-ray diffraction data. It is, however, an overall indicator and thus relatively insensitive to local error in large models. The present study suggests caution in accepting all features of a model solely on the basis that $R$ appears to have reached an acceptable value. In fact, no single value of $R$ can be taken as universally acceptable because the extent and quality of X-ray data from crystals of macromolecules vary widely from one structure to another.

The authors wish to thank Drs E. T. Adman, R. E. Stenkamp and K. D. Watenpaugh for many useful discussions during the refinement work in Seattle. One of the authors (MR) wishes to thank Dr R. Chidambaram for stimulating discussions, Dr M. Srinivasan for his keen interest in the work, and Dr K. K. Kannan for many useful suggestions and help in understanding the *PROTEIN* and *FRODO* program packages. Thanks are also due to Vinay Kumar and S. Charkravarty for interesting discussions. The officers and members of the staff at the BARC, TIFR, and VECC computer centres are thanked for their generous help in making the facilities available. The authors also thank Drs Wayne Hendrickson, T. A. Jones and W. Steigemann for making the *SRLSQ*, *FRODO* and *PROTEIN* program packages available for this work. The refinement in Seattle was supported in part by Grants AM-3288 and GM 34673 from the National Institutes of Health.

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